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The Mast Cell Reaction in Mouse Skin to Some Organic Chemicals*†
III. The Early Effect of Aromatic Hydrocarbons

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(Received for publication August 19, 1947)

In a previous paper (31) the temporary depletion of the granular substance of the dermal mast cells was reported, following the application of benzene to the skin of mice. This reaction failed in response to alcohol, ether, and acetone. Continued studies indicate that a similar reaction on the part of the tissue mast cells can be elicited by some other aromatic hydrocarbons, and is herein described. The subject is limited to the early preneoplastic response of skin to *single applications* of a series of aromatic hydrocarbons.

These hydrocarbons are known to induce a multitude of intermingling reactions on the part of intracellular constituents. Also, they may evoke chemical reactions resulting in the elimination of the original substances or metabolites. Our results draw particular attention to such protective reactions and to the possible role of the granular substance of the mast cells in local detoxication reactions. Our present knowledge of protective reactions to common aromatic hydrocarbons is based chiefly upon feeding experiments (2, 16, 43), and consequently, the part played by different tissue constituents is little known.

Considering the detoxication of aromatic hydrocarbons particular interest is focussed on certain organic sulfur compounds. Cysteine is used for the synthesis of mercapturic acids in response to halogenated benzenes (54) and naphthalenes (7). The glutathione content was found to decrease in the tissue of animals fed naphthalene (37, 42). Phenols, aromatic alcohols and aldehydes conjugate with sulfuric acid to form ester sulfates. The origin of the sulfuric acid is not known (2). Conjugation occurs also in eviscerated and hepatectomized animals (3, 35). Furthermore, glucuronic acid of unknown origin conjugates with a number of compounds, among them hydroxy groups, benzoate, menthols, and naphthalene. Judging from feeding experiments, benzene, phenols, naphthalene, and phenanthrene are partially excreted in conjugation with ethereal sulfates and/or glucuronic acid, and partially converted to mercapturates (2, 7, 16, 37, 42, 43). *In vitro,* the production of phenol sulfate was found to be achieved to a larger extent by rat intestine than by liver, muscle, and kidney slices (1). The interpretation of these findings is hampered mainly by the lack of additional data as to the site of conjugation, the origin of sulfate and glucuronic acid, and the morphology of such tissue reactions.

Pertinent studies by Crabtree on the anti-carcinogenic effect of chemical agents indicate that local protective reactions interfere with the process of skin carcinogenesis. Following the application of naphthalene, phenanthrene, anthracene, and benzene a temporary decrease in the glutathione (G.SH) content of the skin was demonstrated (8-10). This was considered indicative of a local detoxication of the hydrocarbons during the first hours after painting. The G.SH level was restored within 4 hours. Two carcinogenic hydrocarbons did not induce this effect, however (12, 15). The urinary excretion of conjugated ethereal sulfates was increased during the first to fourth days after the local application of benzene, phenanthrene, and naphthalene (10). Attention was paid neither to the morphological tissue changes, nor to the excretion of conjugated glucuronates.

Thus, according to previous investigations, certain sulfur compounds, particularly sulfuric acid, and glucuronic acid play a prominent role in the detoxication of aromatic hydrocarbons, and the origin of these acids is unknown. Now, the point is that the granular substance of the mast cell contains, among other elements, ***heparin,*** which after disin-

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† Because of an accelerated production schedule, the authors have not read proof of this article.

tegration yields considerable amounts of sulfuric acid (sulfur up to 13.6 per cent dry weight; *see* Jorpes [23]), and hexuronic acid (about 26 to 29 per cent; probably glucuronic acid). This is described in detail by Jorpes (23, 29) and Wolfrom and Rice (53). Evidently, the composition of the heparin molecule has extensive bearing on several types of protective reactions. It would therefore be of interest to study the morphological and histochemical reactions of the mast cells to different chemical agents. The present paper will perhaps elucidate one aspect of the functional significance of the mast cell's granular substance.

EXPERIMENTAL

Two series of young and mature male and female Swiss albino mice were used, all on the same basal diet. Standard methods of application and preparations as previously reported were used (30, 31). Painting was done only in the right interscapular skin area, leaving the left side for control. Both methods previously described were used in counting mast cells (30). Following single applications of hydrocarbons to the right interscapular skin of mice of the same age, serial observations were made on the number of mast cells in painted skin flaps and the number of these cells in the symmetrical unpainted control skin areas *in the same animals,* thus assuring the greatest possible accuracy (30). The following symbols are used:

A (experimental) and *a* (control) = the average number of mast cells (60 observations) in a volume of superficial dermis measuring 0.01 mm. × 0.0044 sq. mm.

B (experimental) and *b* (control) = the average number of mast cells (60 observations) in an equal volume of deep dermal and hypodermal tissue.

C (experimental) and *c* (control) = the average number of dermal mast cells (40 to 50 observations) per 1.0 mm. of epidermal length, regardless of dermal thickness (height). Sections 10 microns.

The calculated quotients A/a and C/c are plotted (Figs. 1 to 3, and Fig. 5). The quotient B/b proved to be of minor significance and is therefore omitted in the graphs. For statistical evaluations of results the readers are referred to our preceding papers (30, 31).

Single skin paintings were made using the following aromatic hydrocarbons and derivants:

Benzene	Pure	
Phenol	Cryst.	1% and 2.7% in ether
Naphthalene	Cryst. (Baker)	2.7% in ether
Phenanthrene	Cryst. (Kahlbaum)	2.7% in ether
20-Methylcholanthrene	(Edcan)	0.6% in benzene and acetone

The number of brush strokes will be stated for each series of mice. Because the concentrations and amounts of hydrocarbons used in this series have been kept at levels similar to those reported by Crabtree (10), some comparison may be made of the effects on G.SH level in the skin, but this is not possible with regard to the partition of sulfur in the urines of experimental mice.

A basis for rough estimation of the different concomitant events in the skin of experimental mice was obtained by observing the following changes: initial epidermal cell injury, edema, hyperemia, inflammatory cell infiltration, and the degree of subsequent epidermal regeneration.

RESULTS

I. Mast Cell Reaction to Single Applications of Non-Carcinogenic Hydrocarbons

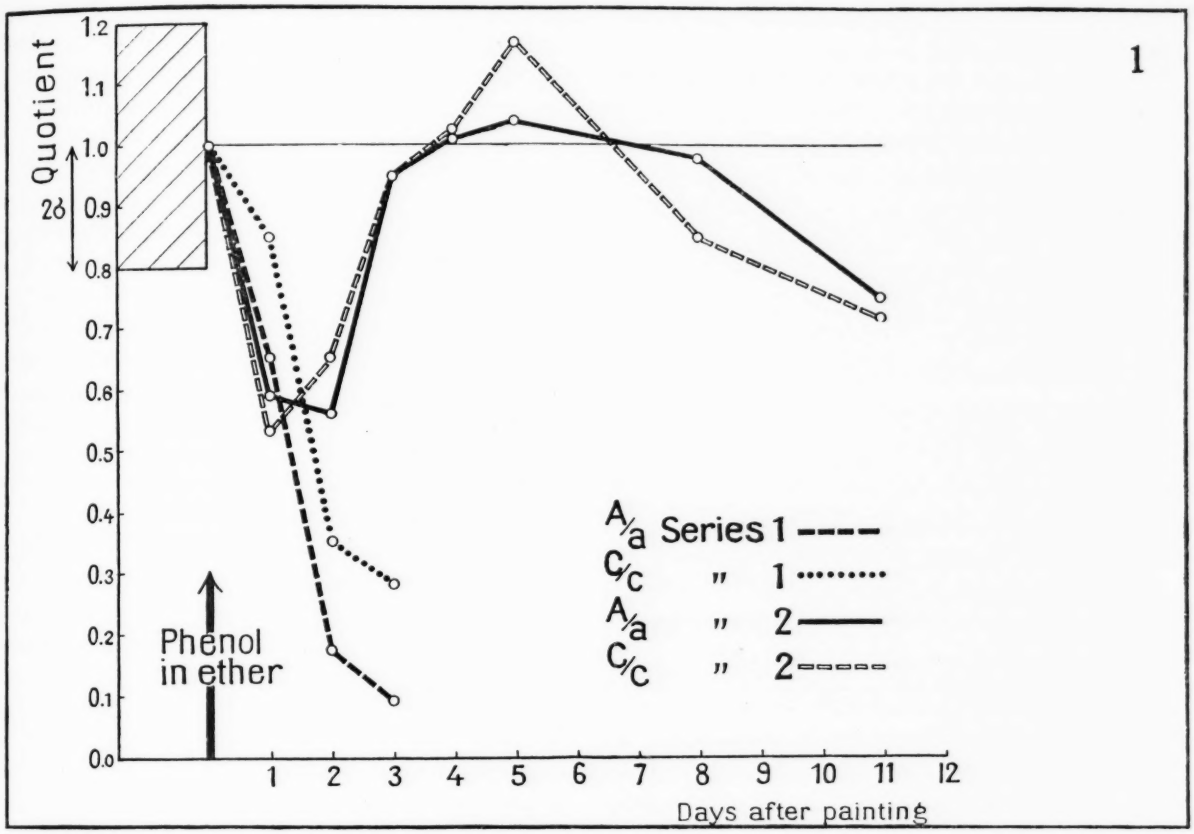
Benzene.—The previously reported depletion of granular substance of mast cells after the application of pure benzene (31) was corroborated (Fig. 4 "a"). Maximum depletion was reached 3 to 5 days after painting, and the normal level was restored after 6 days. Benzene did not cause any significant increase in the number of mast cells during the following 4 weeks. In mature mice, however, the depletion started fairly late (31).

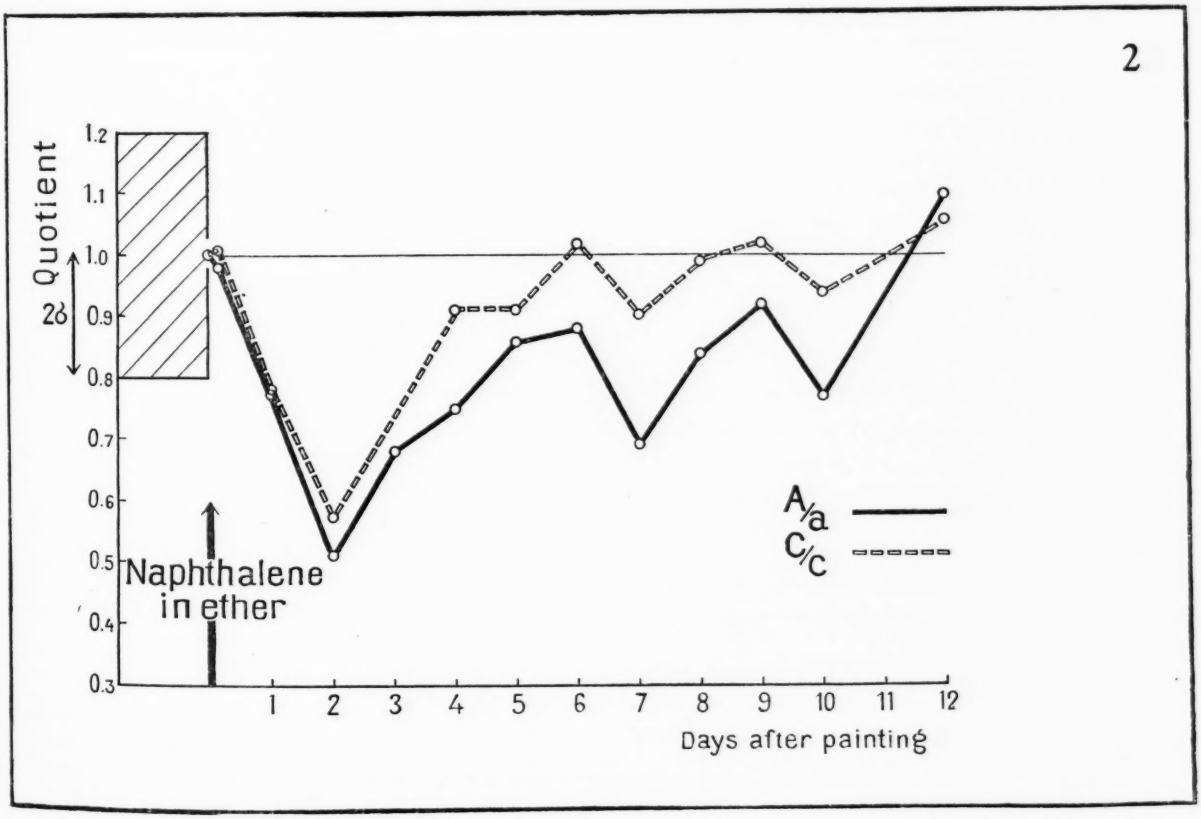
Phenol.—Weak solutions of pure carbolic acid applied to two series of mature mice induced a statistically significant decrease in mast cell quotients (Fig. 1). A moderate drop was observed in response to smaller amounts of phenol (Fig. 1, Series 2), but larger amounts were found to exert a much more pronounced action (Fig. 1, Series 1). Following sublethal doses only about 10 per cent of the normal number of mast cells was observed 3 days later in the superficial dermis. The cor-

DESCRIPTION OF FIGURES 1 AND 2

Fig. 1.—Changes in mast cell quotients after single application of diluted carbolic acid to the right interscapular skin area of mice 6 to 8 weeks old. In series 1 six mice were painted with 4 brush strokes of 2.7% phenol in ether. In series 2 fourteen mice received 2 strokes of 1% phenol in ether.

Fig. 2.—Mast cell quotients after single application of 4 brush strokes of 2.7% naphthalene in ether to the right interscapular skin area of a series of 24 mice 10 to 12 weeks old.


1
Quotient
2δ
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Phenol
in ether
A/a Series 1 ----
C/c " 1 .....
A/a " 2 ——
C/c " 2 - - - -
1 2 3 4 5 6 7 8 9 10 11 12
Days after painting


2
Quotient
2δ
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
Naphthalene
in ether
A/a ——
C/c - - - -
1 2 3 4 5 6 7 8 9 10 11 12
Days after painting

FIGS. 1–2

responding quotients were $B/b = 0.32$, and $C/c = 0.28$.

Naphthalene.—In mature mice moderate amounts of naphthalene destroyed about 25 per cent of the dermal mast cells within 24 hours after painting, and approximately 50 per cent of them 48 hours after application (Fig. 2). The normal mast cell level was again reached in 6 days.

Phenanthrene.—No significant decrease in the number of mast cells could be discerned after single paintings with 2.7 per cent phenanthrene in ether (Fig. 3). Thus, further investigation is needed.

The graphs obtained for benzene, phenol, and naphthalene show a certain uniformity in type and time relationship, and this would seem to indicate the unspecificity in the mast cell response. Roughly, according to the dosage of hydrocarbons a declining order of response is noted, as follows: Phenol, naphthalene, and benzene. Quantitative correlations between dosage of and the depletion of mast cell granular substance could be obtained only for the phenol series. Large amounts of benzene elicit an earlier mast cell reaction than smaller amounts (31), but the level of maximum response remains the same. All graphs indicate the restoration of mast cell balance in a few days without overcompensation, in other words, the primary decrease in granular content did not induce a subsequent increase in the number of dermal mast cells.

II. Mast Cell Reaction to Single Applications of Methylcholanthrene

The early effects of single paintings with 20-methylcholanthrene dissolved in benzene and in acetone were studied in three series of mice (Figs. 4 and 5). When applied to the skin in benzene solution (Fig. 4 "A") a similar response is elicited on the part of the dermal mast cells as to the solvent itself (Fig. 4 "a"). A temporary granular depletion during the first week after painting is followed by a rapid granular regeneration (cp. 31). Three weeks after painting the mast cell level showed a considerable increase in the methylcholanthrene-painted area.

Acetone, it was found, does not induce changes in the mast cell granular content (31), whereas methylcholanthrene applied to young mouse skin in acetone solutions elicits an insignificant decrease in the number of dermal mast cells (Fig. 5), not exceeding twice the standard deviation of quotients (30). No clear-cut correlation was noted

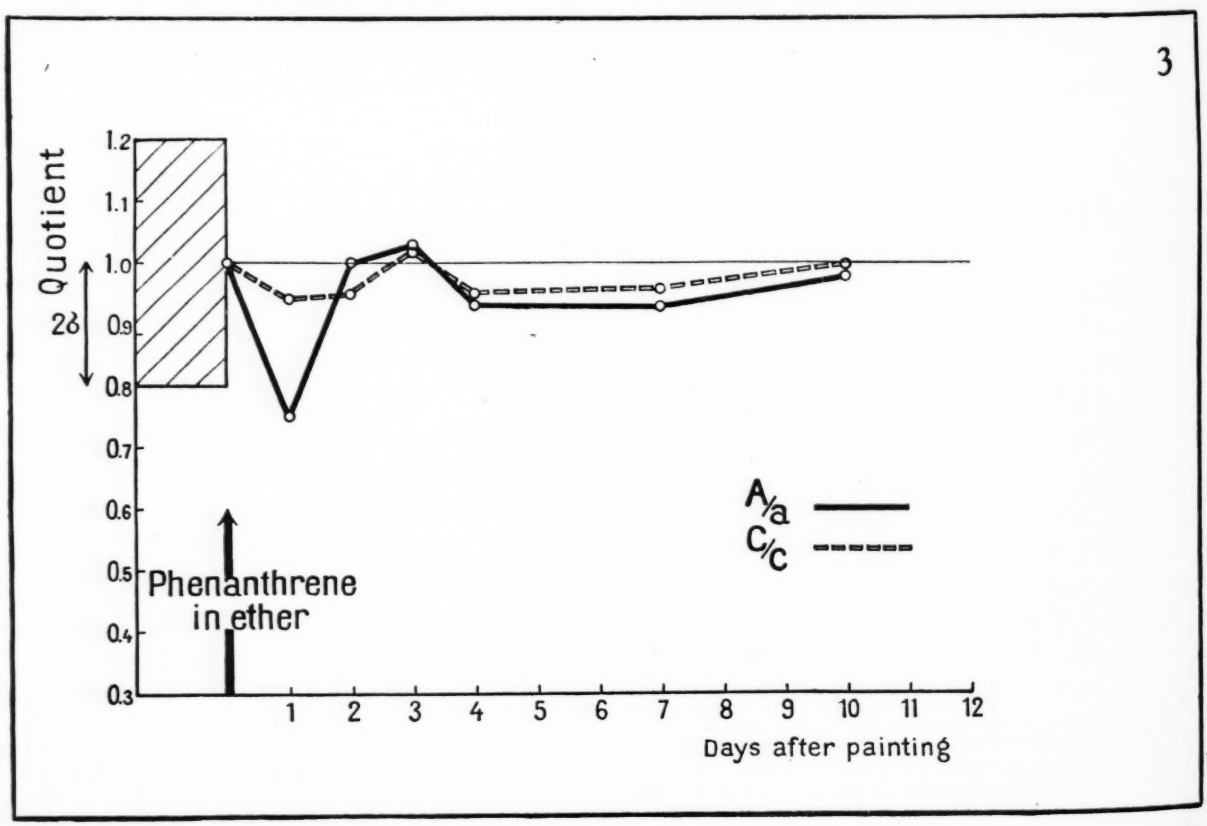


Fig. 3.—Mast cell quotients after single application of 4 brush strokes of 2.7% phenanthrene in ether to a series of 6 mice 6 to 8 weeks old.

for moderate and large doses of the hydocarbon, as shown in Fig. 5, *C/c*. A slight decrease in the number of superficial mast cells (Fig. 5, *A/a*) was interpreted to be as an artefact, due to concomitant edema.

Thus, the mast cell depletion following the application of methylcholanthrene in benzene seems to be attributable to the benzene itself. The carcinogen has so far not induced any apparent early granular decrease in the number of dermal mast cells, nor was a gradient obtained with reference to different dosage. Further investigation with other carcinogenic hydrocarbons is going on. The subsequent increase in the number of dermal mast cells and the cytological changes induced by the carcinogen will be discussed elsewhere.

III. Morphology of the Mast Cell Reaction

Following phenol, naphthalene, and benzene paintings the loss of metachromatic granular substance was gradual until the complete depletion of granules, when the cells became indistinguishable from other connective tissue cell elements. The rate and degree of this reaction varied in different series. During this process both nuclei and cytoplasm increased in size, and a gradual decrease in basophilic affinity of the nuclei was seen. Mast cells deficient in granules had fairly large nuclei with subnormal amounts of chromatin, and pale-staining nucleoli. No abnormal mitoses such as colchicine mitoses, or other degenerative nuclear changes were observed.

The fate of the metachromatic granular substance during this stage could not be clearly explained. Unusual spreading of granules did not occur, nor were mast cells found to move towards the vessels. The usual technical procedures for fixation and staining were employed, but no metachromatic material was immediately discerned in the intercellular fluid of the surrounding connective tissue. Later on, however, small amounts of metachromatic material was demonstrated in the intercellular fluid by means of the "freezing-drying" technic of fixation. This was found after heavy painting with phenol, and seems to be of particular interest. Dissolution of granular material in the mast cell cytoplasm was not seen.

During recovery gradually increasing amounts of small dust-like metachromatic granules were noted in mast cells otherwise quite similar to those just vanished. The nuclei and nucleoli showed an increasing basophilia.

To sum up, we received the impression that the hydrocarbons mentioned above cause a temporary damage to the mast cell cytoplasm and the nuclei resulting in a depletion of cytoplasmic granules, and intranuclear disturbance of chromatin production. To all appearances, these changes are followed by simple recovery of the same cells; resynthesis of cytoplasmic granules, and re-established nuclear functions. The findings do not support the suggestion that the mast cells have been killed or irreversibly damaged during these events. The unusual appearance of metachromatic material in the intercellular tissue fluid indicates that at least a certain amount of granular substance is transferred to this fluid.

IV. Evaluation of Secondary Phenomena

The account of concomitant changes in the skin is of fundamental importance for our interpretations. We must emphasize, however, that our results refer only to *single* paintings. Approximate evaluation of the secondary changes are listed below.

Evidently, no direct correlation could be established between the varying degrees of mast cell response and the subsequent and synchronous secondary changes in painted skin. Neither the initial cell damage, nor the other enumerated secondary changes, such as edema, hyperemia, inflammatory cell reaction, and epithelial regeneration, can be regarded as causative factors *per se*.

V. Possible Interpretations of Results

The results indicate the existence of a non-specific reaction on the part of the mast cell granular substance, induced by the intracellular uptake of some aromatic hydrocarbons and derivatives. After painting the hydrocarbons spread in the intercellular medium and in the cells of the skin according to their ratios of solubility. A good deal of the lower hydrocarbons is rapidly transferred to

	Initial cell damage	Edema and hyperemia	Inflam. cell infiltration	Epidermal regeneration
Benzene, pure	Slight	Moderate	Slight	Slight
Phenol, 1% in ether	Pronounced	Pronounced	Pronounced	"
Naphthalene, 2.7% in ether	Slight	Slight	Slight	"
Phenanthrene, 2.7% in ether	"	"	"	"
Mcha, 0.6% in benzene	Pronounced	Moderate	Moderate	Pronounced
Mcha, 0.6% in acetone	"	"	"	"

the blood,[1] and a part is left in the skin to constitute the active portion. What is taken up by the blood is detoxified elsewhere, and appears in conjugated form or otherwise in the urine.

Regarding the operating mechanisms we must consider the following possibilities.

Cell damage.—Following single applications of the hydrocarbons in this series transitory changes were observed in the nuclei and cytoplasm of the mast cells. These changes might be comparable to those displayed by so-called "mitotic poisons" (32, 33, 34, 39, 40, 41). Our gradation is in accordance with that of Gavaudan (13, 14), who found the mitotic changes to be more specific and intense in response to phenol than to naphthalene and benzene. However, we obtained no reaction with ethyl alcohol, ethyl ether, and acetone, which are known to exert colchicine mitotic efficiency (41). Further, it would be reasonable to assume a marked effect with methylcholanthrene, but the mast cell response failed to appear. In view of these facts it seems to us hardly likely that the mast cell granular reaction should be due primarily to cell damage, but it will be admitted that this mechanism probably is only partially operative.

Chemical effects.—In our opinion the order of hydrocarbon activity with reference to the mast cell granular system, and also the gradients obtained with different dosage of hydrocarbons (phenol and benzene), indicate that the chemical reactivity could be the chief operative factor. The hydrocarbons must induce chemical reactions of protective nature, and the different hydrocarbons in our series demand various chemical groups for their detoxication.

According to investigations by Crabtree (10) a local depletion of G.SH is rapidly induced and restored in the skin. This effect was graded in the following quantitative order: naphthalene, phenanthrene, benzene. This was considered to indicate local mercapturate formation in the skin. Two carcinogenic hydrocarbons (dibenzanthracene and benzpyrene) did not affect the G.SH level. With reference to the present results, this temporary G.SH depletion demonstrated by Crabtree can hardly be regarded as a causative factor.

On the other hand, we must emphasize that all three substances displaying marked activity in our series, namely phenol, naphthalene, and benzene, are known to be rapidly detoxified by conjugation with ethereal sulfates and/or glucuronic acid. Methylcholanthrene does not primarily require any of these compounds, and it may be the reason why this substance does not affect the mast cell granular substance. Two other carcinogens (10) did not affect the G.SH level, nor are they detoxified by simple conjugation (11). Thus, we believe that the reaction of the mast cell granular substance is induced by such hydrocarbons and derivants as require ethereal sulfate and/or glucuronic acid for their elimination. Consequently, *the granular substance of mast cells is supposed to participate in protective reactions.*

Mode of reaction.—The active substances in this series evidently evoke parallel phenomena, such as changes in the physico-chemical state of intracellular structures, and chemical reactions with intracellular constituents. Complex series of events impossible to distinguish in details is predicted. The governing factors can not even be approached because additional data on the local concentrations, solubility, and chemical reactivity, are lacking. Present results indicate, however, that the intracellular uptake of active hydrocarbons is followed by heavy changes of the mast cell granular substance leading to a loss of metachromatic staining reaction. This indicates that the native granular substance either is transferred, or disintegrated with subsequent loss of ester sulfate bonds. The liberation of ester sulfate radicals and hexuronic acid (glucuronic acid) would then constitute prerequisites for detoxication reactions.

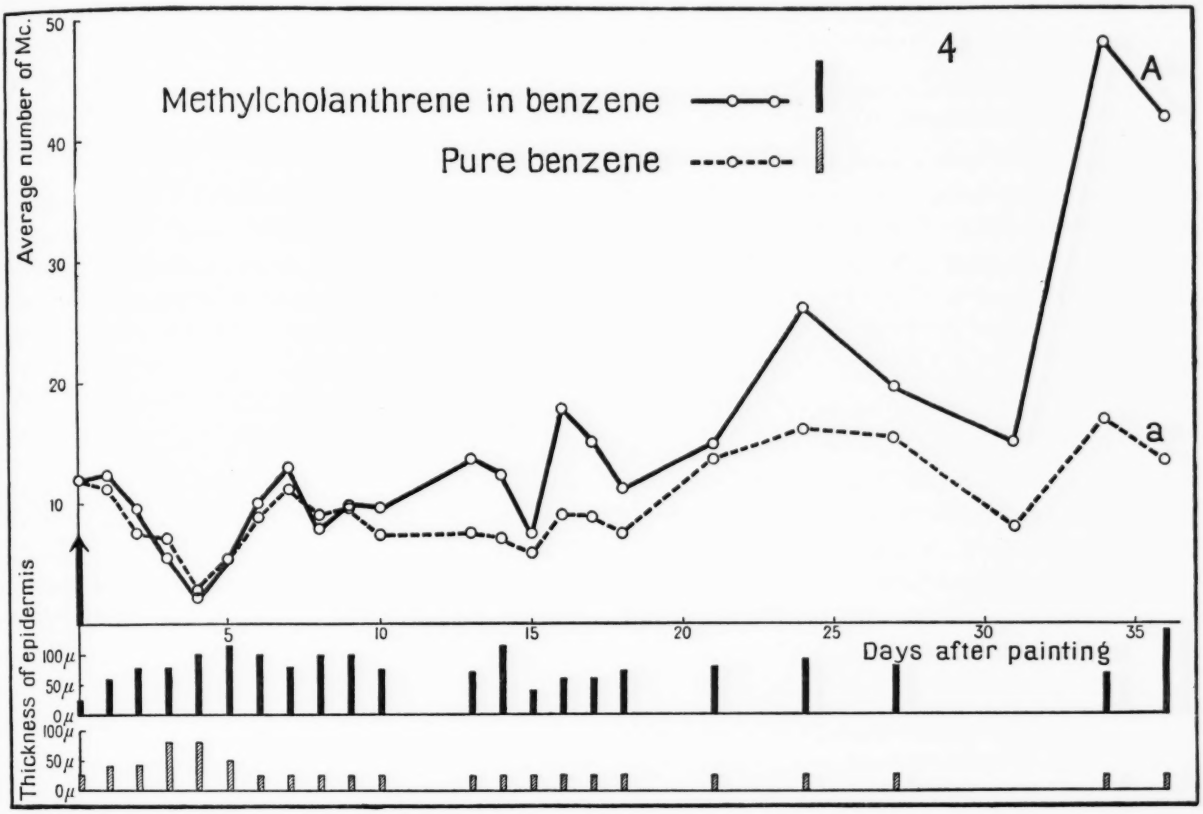
We have as yet no direct evidence favoring the hypothesis that the actual conjugation should occur in the skin. Most of the granular material seems to have disappeared in the cell cytoplasm. A small amount was demonstrated in the surrounding tissue fluid. The conjugation reaction may take place in the mast cells, in the tissue fluid, in the blood, or elsewhere in the body. If, however, the report by

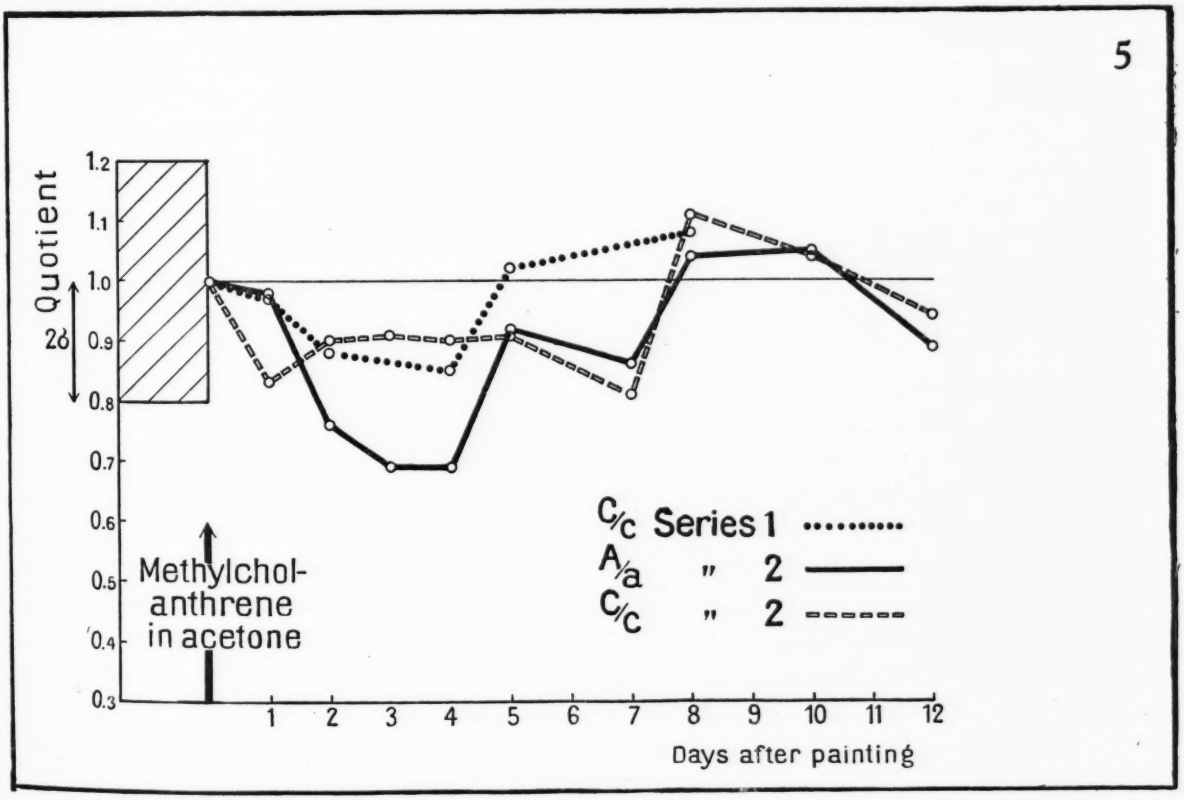
[1]Benzene and phenol cause transient cerebral spasms in mice within a few minutes.

DESCRIPTION OF FIGURES 4 AND 5

FIG. 4.—The numerical changes in dermal mast cells following single painting with 3 brush strokes of 0.6% methylcholanthrene in benzene to the right interscapular region of 22 mice 10 weeks old. For comparison the left interscapular areas in the same mice were at the same time painted with 3 brush strokes of pure benzene.

FIG. 5.—Mast cell quotients following single application of 0.6% methylcholanthrene in acetone. In series 1 six mice (2 weeks old litter mates) were painted with 12 brush strokes. In series 2 fourteen mice 3 weeks old received 3 brush strokes.


4
Methylcholanthrene in benzene
Pure benzene
A
a
Average number of Mc.
50
40
30
20
10
5
10
15
20
25
30
35
Days after painting
Thickness of epidermis
100μ
50μ
0μ
100μ
50μ
0μ


5
Quotient
2δ
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
Methylchol-
anthrene
in acetone
C/c Series 1
A/a " 2
C/c " 2
1
2
3
4
5
6
7
8
9
10
11
12
Days after painting

FIGS. 4–5.

Arnolt and de Meio concerning the conjugation of phenol and ethereal sulfate *in vitro* is recalled, the actual conjugation may occur in the skin as well as in the intestine or liver.

DISCUSSION

This reaction of the granular system of mast cells in response to some hydrocarbons and phenol seems to be the expression of local detoxication procedures in the skin. It provides a basis for further studies in this field, and may explain the origin of considerable amounts of ester sulfate and hexuronic acid (glucuronic acid?) not previously understood. This interpretation was touched upon by earlier authors. Webb (51) found various stages of mast cell granular "dissolution" following intraperitoneal injections of carbon and egg white. The latter substance apparently requires detoxication, but this was not stressed by Webb. The transfer of granular substance (heparin) from hepatic mast cells to the blood during peptone shock in dogs was described by Wilander (52, cp. also 22), and serves as a guide to physiological mast cell function. In this respect, however, his observation seems to be of limited value, although it may indicate a similar detoxication process such as described here.

The present investigations also have a bearing on skin carcinogenesis in general and on the particular line of research dealing with the retardation of experimental carcinogenesis (Crabtree and his associates). It would probably be worthwhile in future discussions on "labile" sulfurous compounds to include not only G.SH, but also the ester sulfates of the mast cell granular system, which to all appearances belong to labile sulfur constituents of connective tissue in general, and may be mobilized in response to different stimuli (10, 13, 45, 48, 50-52).

Regarding the physiological significance of the mast cell granular substance the present situation is rather puzzling and a matter of much controversy (36). As a corollary to the discovery of some chemical components of the isolated heparin molecule (23, 24, 28; cp. also 4-6, 25, 27, 29), Jorpes, Holmgren, and Wilander assume that the tissue mast cells under normal physiological conditions supply heparin to the blood whereas the coagulation of normal blood should be prevented (17-19, 21, 26, 29). They base their hypothesis first, on the conviction that the *native* granular substance *is* heparin, and secondly, on the close perivascular localization of the tissue mast cells (29, p. 63). However that may be, no direct evidence supports this theory. No one has observed the hypothetical delivery of granular substance to the intercellular fluid or to the blood under *physiological conditions*. The perivascular cell distribution may be due to a variety of other factors, *e.g.* a need for high oxygen tension or other matter supplied through the blood.

The physiological function of the mast cell granular substance is still open for discussion. When considering this topic it seems of primary importance to relinquish one-sided, unproven theories (29) prejudicial to further work, and instead keep an open mind toward different lines of approach. The native granular substance probably contains several other biologically active substances (38) and consequently the granular system may have some bearing on *several* types of biological reactions. A number of observations indicate a close relationship between the granular substance and different growth processes (44-50), but the underlying mechanism is as yet not fully understood (50). Secondly, according to the present investigation, the granular substance seems to take part in local detoxication reactions.

SUMMARY

1. Following single applications of benzene, phenol, and naphthalene to mouse skin the early depletion of granular substance on the part of dermal mast cells was reported. This effect was not obtained by using phenanthrene or 20-methylcholanthrene.

2. The agents cause reversible damage to the intracellular structures of mast cells.

3. In an attempt to interpret these findings the concomitant secondary changes could with some certainty be ruled out as operative factors. The interpretation is advanced that some constituents of the granular substance, *viz.* ethereal sulfate and hexuronic acid, take part in detoxication reactions aiming at the elimination of the agents under question. Continued investigations are needed for the elucidation of the operating factors.

4. It is concluded that the native granular substance of tissue mast cells has some bearing on local detoxication reactions as well as on other types of biological reactions.

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DESCRIPTION OF FIGURES 6 TO 11

FIG. 6.—Unpainted left control skin area showing the normal distribution of mast cells. Toluidine blue. Mag. × 325.

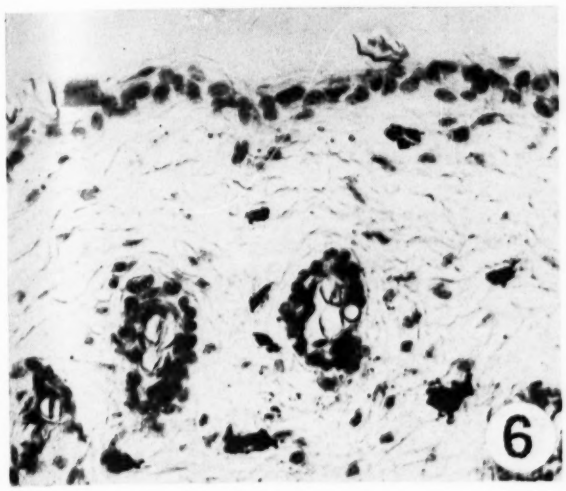
FIG. 7.—Three days after one single application of phenol 2.7 per cent in ether almost all granule-bearing mast cells have disappeared. Epidermal necrosis, marked dermal inflammatory cell infiltration. Same animal as in Fig. 6. Toluidine blue. Mag. × 325.

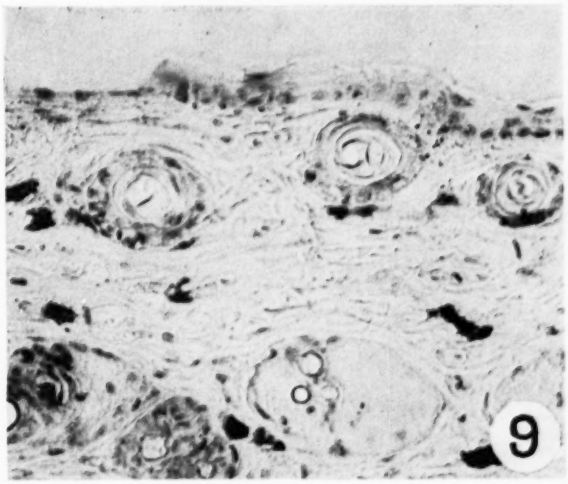
FIG. 8.—Same as Fig. 7. V. Gieson. Mag. × 325.

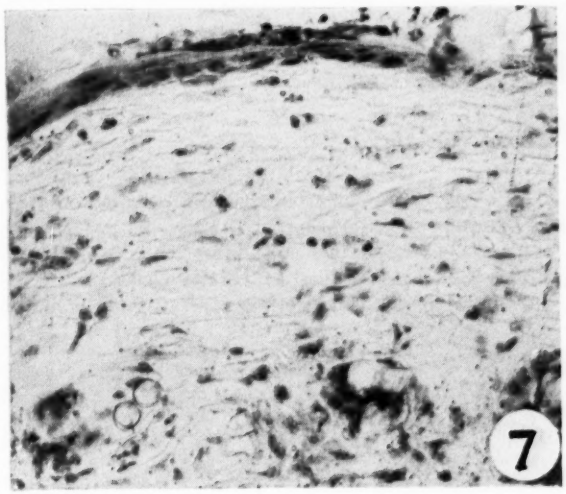
FIG. 9.—Unpainted left control skin area from a naphtalene treated mouse. Normal number of mast cells. Toluidine blue. Mag. × 325.

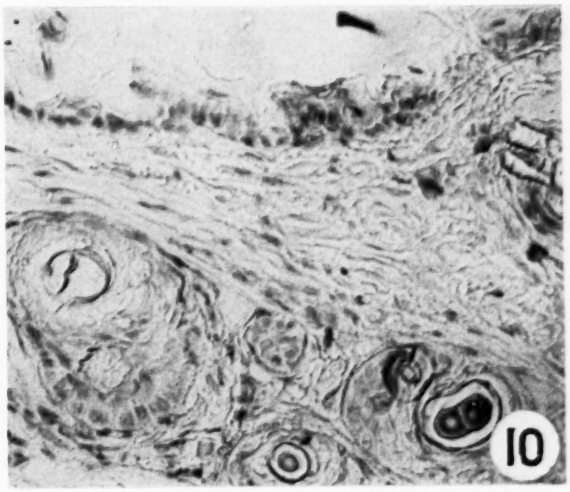
FIG. 10.—Two days after one single application of naphthalene 2.7 per cent in ether. Almost all superficial dermal mast cells have disappeared, and the number of hypodermal mast cells has decreased considerably. Toluidine blue. Mag. × 325.

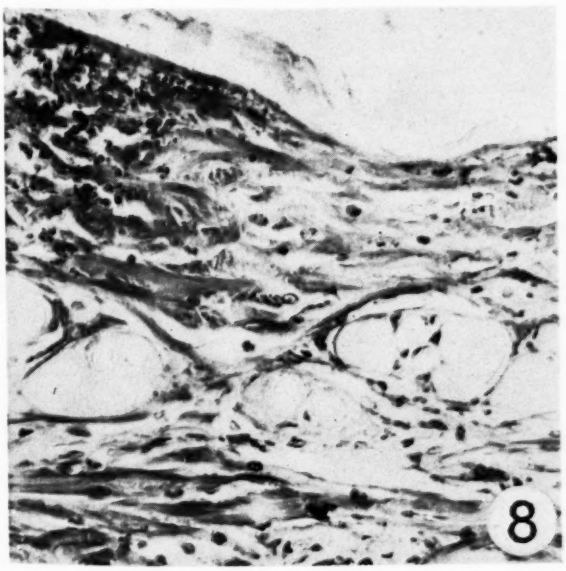
FIG. 11.—Same as Fig. 10. No conspicuous cell changes are seen; only slight inflammatory symptoms. V. Gieson. Mag. × 325.

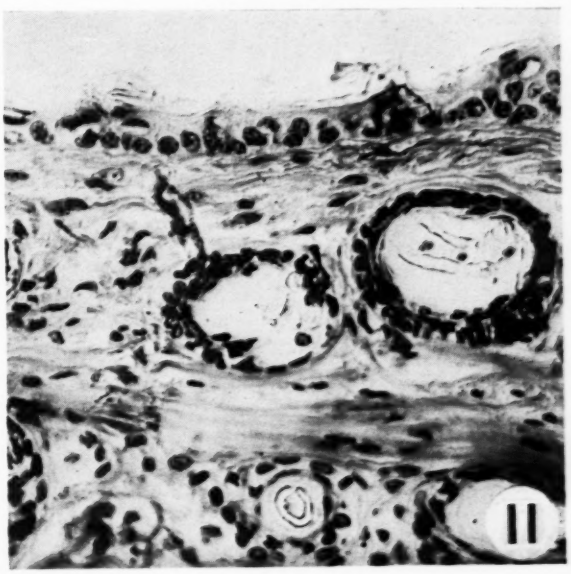












FIGS. 6-11

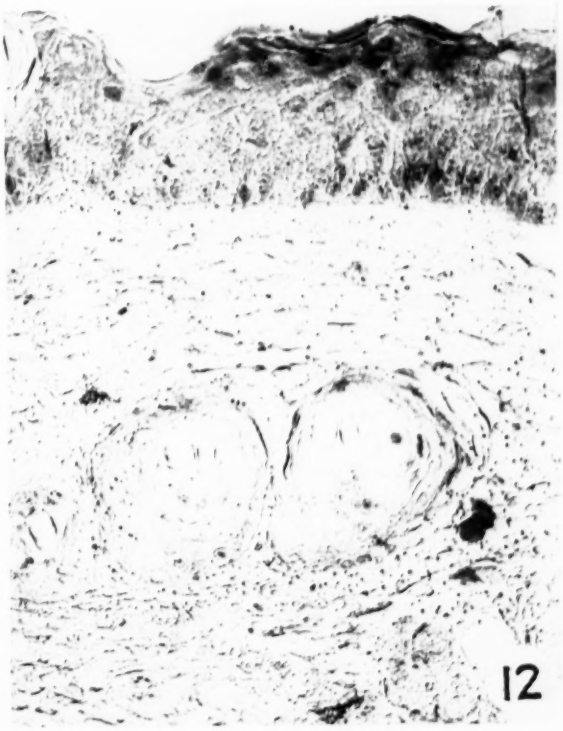
DESCRIPTION OF FIGURES 12 TO 15

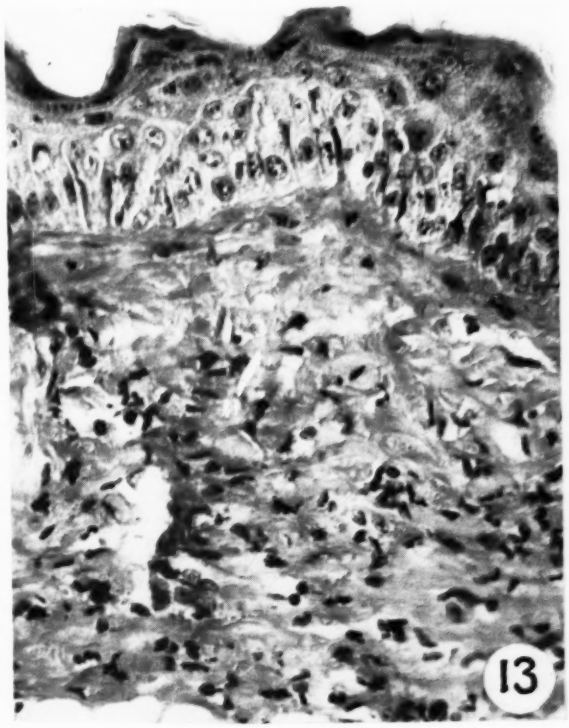
FIG. 12.—Three days after a single painting with methylcholanthrene 0.6 per cent in benzene. A marked depletion of superficial mast cells; some are left in the deeper part of dermis and in the hypodermis. Toluidine blue. Mag. × 325.

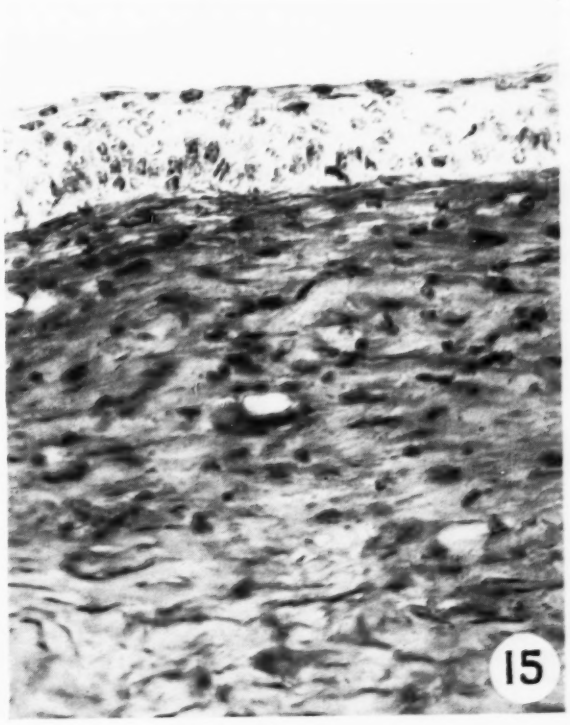
FIG. 13.—Same as Fig. 12. The changes in epidermal texture and cytology are seen, as well as edema and cell infiltration in dermal connective tissue. V. Gieson. Mag. × 325.

FIG. 14.—Ten days after one single application of methylcholanthrene 0.6 per cent in benzene, a normal number of mast cells is reattained. Toluidine blue. Mag. × 325.

FIG. 15.—Same as Fig. 14. Marked changes are still seen in both epidermal and dermal layers. V. Gieson. Mag. × 325.







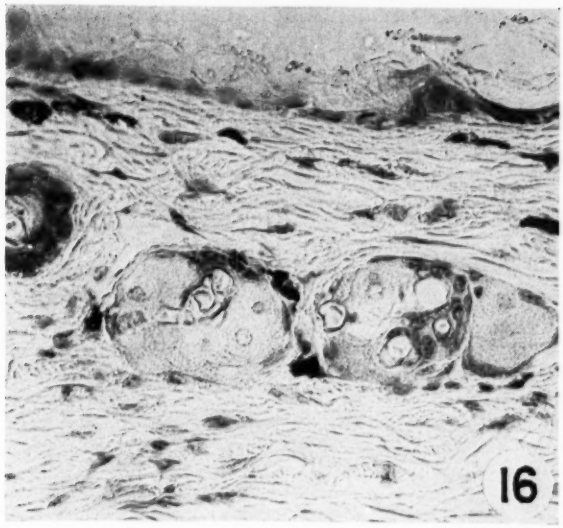
FIGS. 12-15

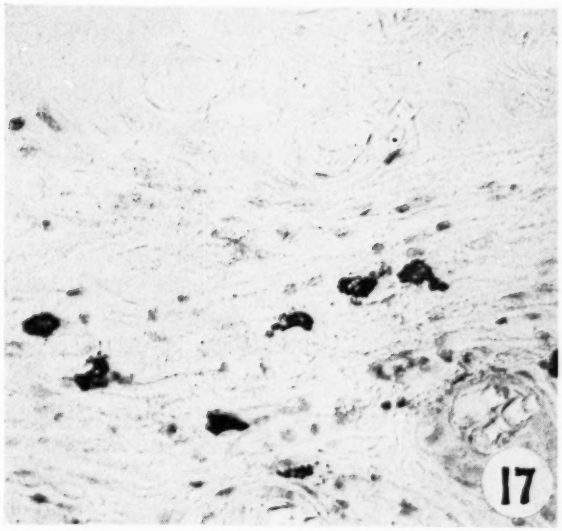
DESCRIPTION OF FIGURES 16 TO 18

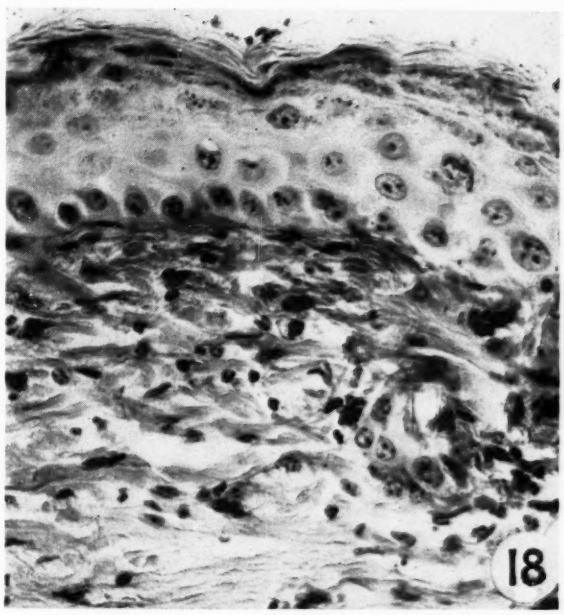
FIG. 16.—Unpainted left control skin area from the same methylcholanthrene treated mouse as in Fig. 17. Normal number and distribution of mast cells. Toluidine blue. Mag. × 325.

FIG. 17.—Four days after a single application of methylcholanthrene 0.6 per cent in acetone. A slight decrease in the number of superficial dermal mast cells is seen. Toluidine blue. Mag. × 325.

FIG. 18.—Same as Fig. 17. Marked epidermal nuclear changes, and dermal edema and inflammatory cell infiltration. V. Gieson. Mag. × 325.







FIGS. 16-18

Growth in Tissue Culture of Analogous Mouse Mammary Carcinomas and Their Response to Radiation

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The mammary tumors used in these experiments were from two inbred strains of mice, dba and C3H, in both of which a high incidence of mammary tumors occurs.

The dba strain has been inbred in the Roscoe B. Jackson Memorial Laboratory since 1918. The mammary tumor, designated dbrB, which arose in this strain was histologically diagnosed as an adenocarcinoma. It is a fast growing tumor which, implanted into hosts of the same strain, results in 100 per cent "takes" with a latent period of 4 to 6 days, and kills the host within 3 to 4 weeks.

The C3H strain of mice was established by Dr. L. C. Strong and has been inbred since 1920. The tumor occurring in this strain, also histologically diagnosed as an adenocarcinoma, results in 95 to 100 per cent "takes" when implanted within hosts of the same strain. It is a relatively slow growing tumor, with a latent period of 12 to 15 days.

Figs. 1 and 2 are sections of the two tumors, both showing typical alveolar glandular structures. Numerous mitoses, which occur in both, are best shown in Fig. 1.

In a previous study by one of us (3) it was found that these two analogous tumors, in hosts of their respective strains, differed widely in their radiosensitivity. Thus, implants of the dbrB tumor of the dba strain, required a dose of about 5,000 r to prevent "takes" while implants of the C3H strain required only about 2,700 r.

The latent periods of the irradiated implants of the respective tumors varied significantly. For example, the longest latent period of an implant of the dbrB tumor exposed to a threshold dose of 4,500 r was 54 days, while that of the mammary tumor of the C3H strain exposed to a threshold dose of 2,600 r was 38 days. It appears, therefore, that the ability of the tumor-implants to establish themselves in their respective hosts, and to recuperate following radiation are different.

The question arises: Does the difference of these two tumors in their rate of growth and in their response to radiation depend upon specific characteristics of the tumors or does it depend upon the host? In order to obviate confusing factors in the living animal, the present study using the tissue culture method, was undertaken. This should offer information on the qualities of the tumor cells *per se*.

EXPERIMENTAL PROCEDURE

Actively proliferating tumors were aseptically excised from the animals and peripheral portions cut into fragments of about 4 to 5 mm. Five or six fragments were placed on a coverslip, previously attached by a film of fluid to a mica sheet. The total thickness of the coverslip and mica was about 0.25 mm. The mica carrying the coverslip and tissue fragments was inverted over a depression slide in which moist filter paper had been placed to maintain adequate moisture of the tissue. The tumor fragments so prepared were immediately exposed to x-radiation under an oil-cooled pulsating potential Coolidge tube. Doses of from 5,000 r to 130,000 r were given.

The radiation factors were: 200 KV., 0.5 mm. Cu plus 1.0 mm Al filtration, and H.V.L. of 0.9 mm. Cu. The tumor fragments were irradiated at 12.5 cm. distance from the target, and the average intensity was 604 r per minute. In calculating the dose of x-rays delivered to the tumor fragment, the absorption of the radiation by the covering glass and mica sheet, which is about 10 per cent, was taken into consideration. The dose of radiation in each case was applied by a single exposure.

It should be noted that in this study the exposure to radiation was made on the tumor fragments and not, as commonly done, in a tissue culture, on sheets of growing cells already imbedded in a clot.

This was done because one of us (4) had found that a dose of radiation produced a greater effect on fragments remaining in the tissue culture medium in which they had been exposed than when they were removed, washed in Tyrode solution,

and imbedded in a fresh culture medium. An explanation of this is that, upon irradiation, substances appear in the medium which are toxic to the tissue. Similar observations have been made by other investigators (2, 6). In addition to this, the irradiation of complex tissue fragments, instead of cells cultured *in vitro,* was considered preferable since this type of tissue had been used in the experiments *in vivo* and the results would therefore be more comparable.

Within 30 to 50 minutes after irradiation, the tumor fragments were carefully washed in Tyrode solution, after which they were cut into pieces of about 1 cu. mm. These pieces were then placed in a tissue culture medium consisting of a mixture of fowl plasma, rat serum, and chick embryo extract, sealed over a depression slide and incubated at body temperature. The same technic was used for the control non-irradiated fragments.

The cultures were examined daily and maintained for 7 to 9 days by washing in Tyrode solution and adding fresh medium every 48 hours. Some cultures of each series were fixed in Carnoy's solution every second day and stained with hematoxylin and eosin.

Cultures of non-irradiated mammary tumors of the C3H and dba strains.—The non-irradiated fragments of both tumors grew quickly and luxuriantly. The explants from the C3H strain produced dense, compact sheets of epithelium several layers thick with a few columns of tubular structure. Very active macrophages were numerous and migrated far in advance of the out-growing epithelial sheets. Some fibroblasts were seen and mitotic figures were present (Fig. 3). The explants from the dbrB tumor grew more rapidly and within 24 hours produced sheets of cells which tended to be more loosely held together; mitoses were more frequent and fibroblasts and macrophages were fewer, than in the cultures of the C3H strain (Fig. 4).

Cultures of irradiated tumor of the C3H strain.—Exposures of 5,000 to 15,000 r did not appreciably affect the rate of growth. Within 24 hours, new epithelial sheets were formed around the explants. These gradually increased in the next 5 to 9 days; however, the epithelial sheets were slightly thinner than those of control cultures. Some macrophages and fibroblasts were present in the outgrowth although somewhat fewer than in the control cultures.

With a dose of 20,000 r inhibition of growth was first noticed. Only about 50 per cent of the irradiated explants produced sheets of epithelium within 24 to 48 hours' incubation while the remainder produced new sheets of epithelium within the next 7 days. No wandering cells or fibroblasts were observed in any of these cultures. The effect of this dose was manifested in the delayed epithelial growth and in the total destruction of the macrophages and fibroblasts. As is well known, and has been shown in tissue culture (1, 5), there is a selective action of radiation on different types of cells, and a greater sensitivity of wandering cells and fibroblasts to radiation.

With doses of 30,000 and 40,000 r, the latent period was slightly longer. Epithelial sheets appeared in less than 50 per cent of the cultures within 24 to 48 hours, and in the remaining explants within the next 7 days. The epithelial sheets were thinner and smaller, although the cells appeared normal and the majority had well defined nuclei. Occasionally, a cell was noticed with an abnormally large nucleus.

A dose of 50,000 r delayed the growth of 80 per cent of the explants until the fourth day. However, by the seventh day, all the explants showed good growth but with some variation in the shape and size of the nuclei.

With exposures of 100,000 r and 110,000 r, only 25 per cent of the explants produced growths within 7 days. Although 100,000 r did not appreciably inhibit epithelial growth and most of the cells appeared normal, the variation in the size and shape of the nuclei typical of this type of tumor was increased, and an occasional vacuole occurred in the cytoplasm (Fig. 5).

Exposure to 130,000 r prevented the implants from producing any new growth.

Cultures of irradiated tumor dbrB of the dba strain.—No consistent effects were produced with dosages up to 20,000 r. On the other hand, all the cultures of explants exposed to a dosage of 20,000 r produced extensive sheets of epithelium. These, however, were loose and the cells somewhat irregular in size and shape, manifesting general signs of degeneration. A large number of cells were vacuolated with fragmented nuclei, while stained preparations showed the cytoplasm markedly eosinophilic. Throughout the cultures, chromatin particles could be seen which were identified as the "chromatin dust", known to be produced by effective dosages of radiation on the nucleus. A few wandering cells and a few fibroblasts were also present. The effect of the radiation is shown in Fig. 6.

Similar observations were made with a dose of 30,000 r. However, the degenerative changes were

increased, with more cells of irregular shape and size, and more vacuolation.

With the increase of the x-ray dosage a drastic decrease in the percentage of growing explants occurred, and degenerative changes were more noticeable. Explants exposed to 50,000 r produced epithelial sheets, but the cells were vacuolated, the nuclei broken up and the chromatin substance clumped. Exposure to 60,000 and 80,000 r allowed growth of about 75 per cent of the explants. The new growth, however, consisted of loosely arranged single cells, instead of intact sheets. The shape and size of the cells differed markedly from those of non-irradiated explants. In some areas only huge nuclei without any distinguishable cytoplasm were seen. This is shown in Fig. 7. Explants exposed to 100,000 r showed no growth.

A comparative analysis of the results of the growth of these two analogous tumors, gives evidence in tissue culture that the tumor of the C3H strain is more resistant to x-rays than the dbrB tumor of the dba strain.

DISCUSSION

From previous work it has been shown that two adenocarcinomas in the C3H and dba mouse strains, respectively, though histologically identical, differ in their radiosensitivity. Thus, it was found that the tumor of the C3H strain, planted *in vivo,* was more adversely affected by having been irradiated than the dbrB tumor, of the dba strain. This report presents an additional significant finding, that the differential susceptibility of the irradiated fragments is reversed in tissue culture. Thus, when the irradiated fragments are grown in tissue culture, it is dbrB which is more adversely affected than C3H.

In tissue culture, the effect of the host is obviously absent. Hence, we might legitimately infer from the results of the tissue culture experiments that the dbrB tumor of the dba strain is far more radiosensitive than the tumor of the C3H strain.

The question arises why a tumor which, from the evidence of tissue culture is more radiosensitive should, when planted *in vivo,* be more radioresistant.

It is known that the establishment and growth of the tumor implant in the host depends upon the ability of the tumor to elicit adequate vascular supply, whereas in tissue culture the nutritive supply is already present. One may speculate that the mechanism of the mammary tumor of the C3H strain to elicit vascular supply is more readily impaired by radiation than that of the dbrB tumor.

The cells of the two tumors may appear to be morphologically similar. However, their physiological response following irradiation must be very different according to whether they are in conjunction with or apart from the host.

The tissue culture method reveals the intrinsic properties of cells unobscured by variable host reactions.

Hence, it is incorrect to conclude from effects *in vivo* that a given tumor is or is not intrinsically radiosensitive, since the physiological characteristics of the host evidently play so large a part in the behavior of irradiated tumors.

SUMMARY

A comparison was made of the growth characteristics *in vitro* of two analogous mammary tumors of inbred strains of mice, C3H and dba, and the relative response of these tumors to radiation. It was found that although histologically similar, their reaction to radiation not only differed, but when grown *in vitro* gave the reverse reaction to that when grown *in vivo*. Thus, a dose of about 5,000 r was required to prevent the proliferation of implants of the dbrB tumor when grown *in vivo,* whereas a dose of about 2,700 r was sufficient to produce the same effect on implants of the mammary tumor of the C3H strain. From studies *in vitro,* the reverse was noted; thus, a dose of 20,000 r produced significant changes in the cells of the dbrB tumor, whereas a dose of 100,000 r still left the majority of the cells of the mammary tumor of the C3H strain intact and without any apparent effect either on the nucleus or on the cytoplasm.

In the light of observations made from this study, one may infer that certain intrinsic factors exist independent of histological structure, which govern the growth of a tumor and its response to radiation.

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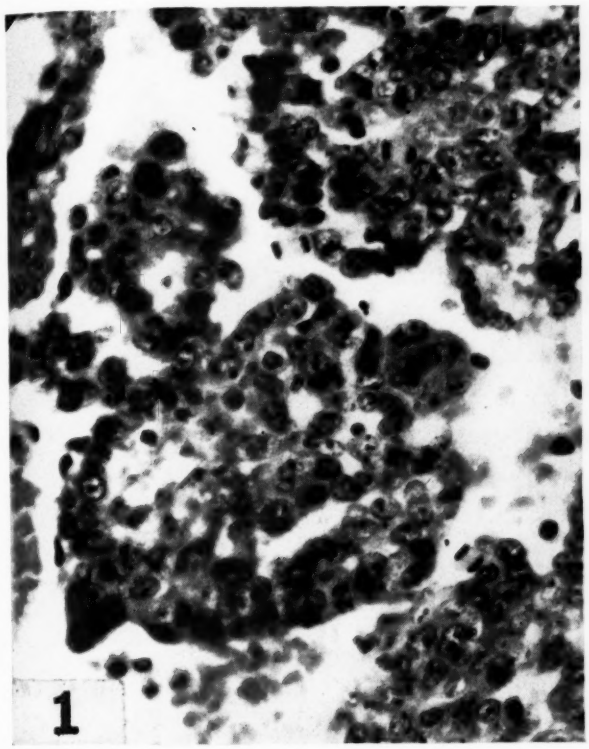
DESCRIPTION OF FIGURES 1 TO 4

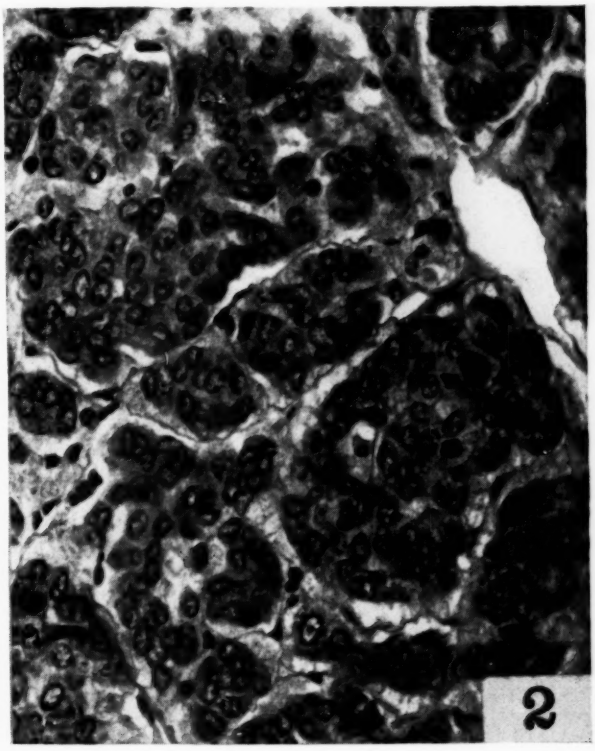
FIG. 1.—Section of the dbrB tumor. Note acini and mitotic figures. Mag. × 473.

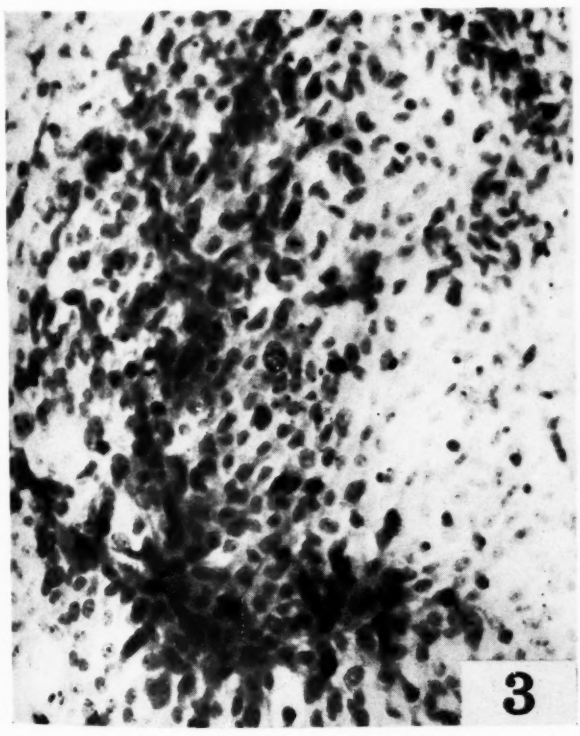
FIG. 2.—Section of mammary tumor of the C3H strain. Note acini, which are more compact, and mitotic figures, which are less, than those of Fig. 1. Mag. × 473.

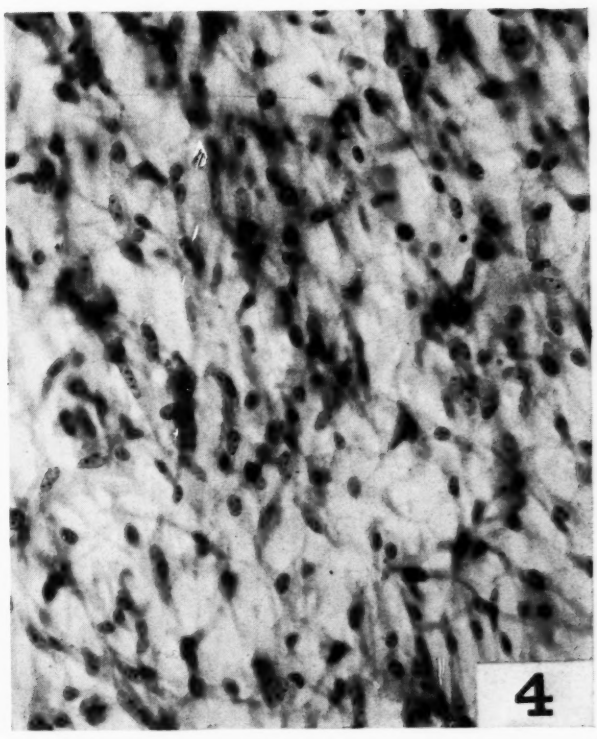
FIG. 3.—Control culture of the mammary tumor of the C3H strain. Note dense growth of epithelial sheets, mitotic figures, infiltrating round cells, macrophages, and some fibroblasts. Mag. × 200.

FIG. 4.—Control culture of the dbrB tumor. Note sheets of epithelial cells, which are looser than those of Fig. 3, mitotic figures, some infiltrating round cells, and fibroblasts. Mag. × 200.









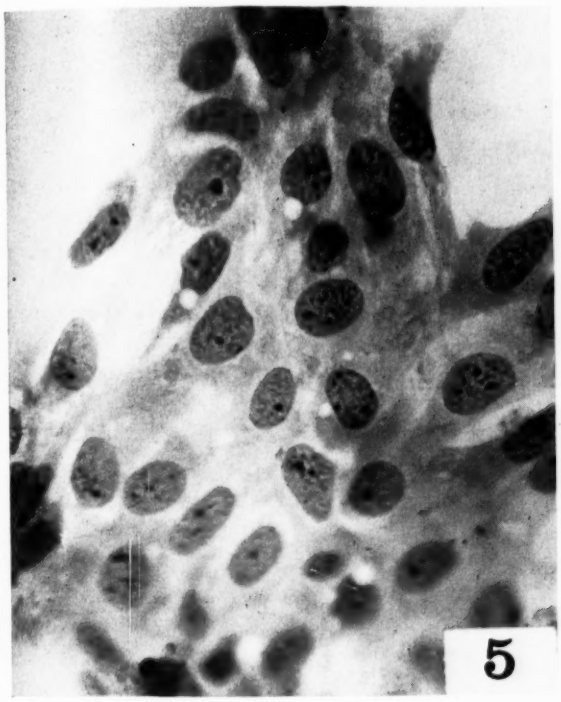
FIGS. 1-4

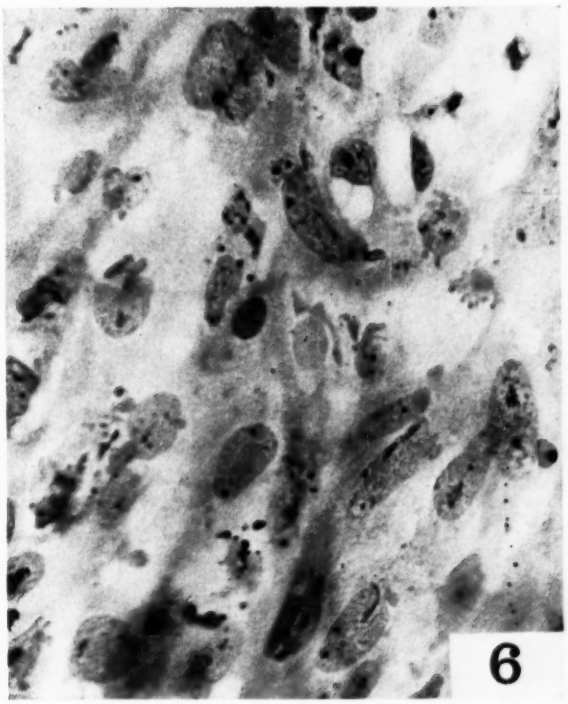
DESCRIPTION OF FIGURES 5 TO 7

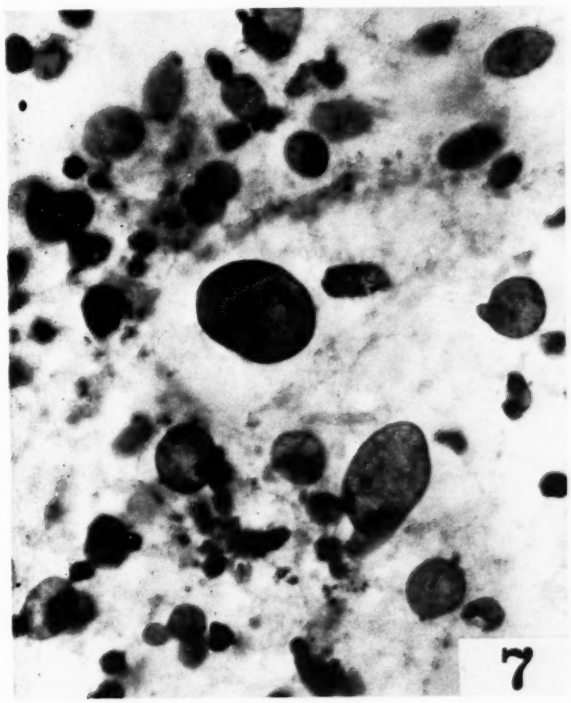
FIG. 5.—Culture of explant of mammary tumor of the C3H strain, exposed to 100,000 r. Note the sheets of epithelial cells, the variety in size and shape of nuclei; some vacuoles in the cytoplasm. Mag. × 1,076.

FIG. 6.—Culture of explant of the dbrB tumor exposed to 20,000 r. Note sheets of epithelial cells, variation in shape and size of nuclei; a number of cells are vacuolated, with broken-up nuclei; chromatin particles throughout the field. Mag. × 1,076.

FIG. 7.—Culture of explant of dbrB tumor exposed to 80,000 r. Note the great variety in nuclear size, the conspicuous large round nuclei; cytoplasm not distinguishable. Mag. × 1,076.







FIGS. 5-7

A Common Mesenchymal Tumor of the Corium of Goldfish, *Carassius auratus**

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The tumors that commonly arise in the corium of goldfish possess characteristics that are useful in several types of investigations concerned with problems of neoplasia: (a) They tend to be prevalent among the fishes of certain pools, whereas they are rare or absent in the population of others. Where prevalent, the number of tumor-bearing fish as a rule gradually increases. Evidence will be presented that the factors inducing the tumors are environmental rather than hereditary. (b) The adaptibility of goldfish to laboratory conditions and the exposed position of the tumors permit study of the natural history of the neoplasms, particularly with respect to their inception and course. Thus it has been learned that in their early stages some of the tumors regress and disappear, while others become established and exhibit progressive growth. Such behavior suggests that the inception of these tumors and their further development depend on different factors.

Despite the high incidence of these neoplasms in the populations of certain pools, there are only 13 reports of tumors in goldfish on record; these are summarized in Table 1. It will be noted that all but 3 reports concern tumors arising either in the corium or subcutaneously from mesenchymal elements. The authors have classified the growths as fibromas or fibrosarcomas. Judging from the description and illustrations, the previously recorded neoplasms are of the same kind as those dealt with in this paper. The small number on record is surprising; it probably does not indicate rarity of these tumors, but rather that they have hitherto attracted but little attention.

In the present paper we shall, first, describe the gross and microscopic appearance of these neoplasms in 30 individuals; second, report our observations on the course of the tumors; and, third, give an account of experimental attempts to transmit or transplant the neoplasms. The methods employed in the experiments will be given under the separate headings.

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Gross appearance and regional distribution of the tumors.—The appearance of representative tumors is shown in Figs. 1 to 6. The majority of the tumors were located on the side of the body (Figs. 1, 2 and 4), the operculum (Fig. 5) or the head (Fig. 6); 7 partly covered the sclera and cornea (Figs. 4 and 5); 3 affected the tail fin (Fig. 3), a number of small growths the other fins. The distribution of the tumors is graphically shown in Figs. 7 and 8. It will be noted that there was no favored site of origin, excepting that none of the tumors was located on the ventral surface. The tumors projected outward; they usually had an hemispherical or oval shape, and were flattened at the base. The surface was generally smooth, though occasionally ulcerated, and always devoid of scales. The color was ivory-white or very pale yellow. In size, the tumors ranged from a few millimeters to over 3 cm. More specifically, in 16 fish the largest tumor measured less than 10 mm.; in 14 fish the size varied from 10 to 35 mm. Since the overall length of the 30 tumor-bearing fish was, on the average, only 15 cm., the majority of the neoplasms were relatively large. Thus, the massive tumor shown in Fig. 1 was approximately one-third the size of the entire body.

The smaller tumors were usually fairly firm in consistency, the larger were soft. The cut surface was pale to faintly pink, moist, and occasionally bloody; no definite pattern of structure could be recognized grossly. None of the tumors was encapsulated, but all of the smaller growths were fairly well circumscribed. Only the larger growths infiltrated the subjacent tissue.

The tumors were solitary in 23 fish; the remaining 7 fish had 2 or more tumors. When multiple, the tumors probably were incited by the same factors acting on more than one focus.

No visceral metastases were found, even though about one-half the tumors were locally infiltrating. Previously one of us (17) had reported a goldfish with 3 subcutaneous "fibrosarcomas"; the two smaller growths were considered as metastases from the larger tumor; this interpretation was probably erroneous. The only instance of possible

TABLE I: TUMORS OF GOLDFISH REPORTED IN THE LITERATURE

Authors' classification of tumors	Site	Number of cases reported	Number of tumors per fish	Experimentation	Author	Date
Spindle cell sarcoma	Dorsal fin	1	Solitary	———	Bland-Sutton	1885
Sarcoma	?	1	"	———	Semmer	1888
Carcinoma	Bladder	1	"	———	Plehn	1909
Fibroma	Orbit	1	"	———	Guglianetti	1910
Fibrosarcoma	Subcutaneous on back and near tail	1	3 tumors	———	Schamberg and Lucké	1922
Myxofibroma	Skin of body, fins and eyes	Several	Multiple	———	Wago	1922
Fibrosarcoma	Subcutaneous at base of tail	1	Solitary	———	Johnstone	1923
Lymphosarcoma	Kidney	1	"	———	Plehn	1924
Fibrosarcoma	Subcutaneous	7 (all in same tank)	"	After removal of tumor-bearing fish and disinfection of tank no new cases appeared	Ruffo	1924
Fibroma	Skin	1	Multiple	———	Sagawa	1925
Papillary epithelioma	Pectoral fins	2	2 tumors	———	"	"
Fibroma	Subcutaneous	1	Solitary	Transplantation to other goldfish was unsuccessful	Eguchi & Oota	1926
Fibrosarcoma	Dorsal fin	1	"	———	Dominguez	1928
Fibrosarcoma	Subcutaneous and on fins	5 (all in same pool)	"	Transplantation to 9 goldfish was unsuccessful	Montpellier and Dieuzeide	1932

metastases from goldfish tumors of the kind with which we are dealing here is recorded by Dominguez (3); in his case nodules regarded by the author as metastases were present in the liver and the swim bladder.

Microscopic appearance.—The tumors arise in the corium. This layer in goldfish, as in other fishes, is mainly composed of connective tissue, which varies in thickness in different regions. It is loosely textured in the outer, and more compact in the deeper portion; the former carries most of the small blood vessels and nerves, many pigment cells and perhaps some muscle fibers. It is this layer which probably gives origin to the tumors.

In their general appearance the tumors resemble neoplasms of connective tissue. There is some difference in structure between the smaller, more slowly growing tumors, and the larger, growing more actively.

The smaller tumors are composed mainly of elongated cells which are arranged in interlacing fasciculi, so that in sections the cells of neighboring bundles are cut in different axes. The cell outlines are usually indistinct (Figs. 9 to 13). The cytoplasm varies in amount from scanty to abundant; in some cells it is faintly granular; in others, distinctly fibrillated. The nuclei are oval in shape and usually have well rounded rather than pointed ends; they are relatively small, fairly uniform in size, and stain deeply (Fig. 12). Occasionally there is a suggestion of a pallisading alignment (Fig. 13). Mitotic figures are few. When stained with Masson's trichrome mixture, delicate blue fibers are seen between the cells in some but not in all regions; the fibers usually run in the long axis of the cells and show little tendency to wrap themselves around them. Silver stains (Wilder's, Perdrau's) bring out a network of reticulum, which, like collagen, is nowhere abundant and in some areas entirely absent. The smaller tumors are not invasive, though they are not encapsulated.

In contrast, the larger neoplasms are distinctly invasive and extend for a variable distance into the subjacent muscle (Fig. 15). Their cells are more pleomorphic (Figs. 14, 16 and 17), relatively large, and with an abundance of cytoplasm and prominent hyperchromatic nuclei (Figs. 18, 19 and 20). Many are multinucleated giant cells, the nuclei forming dense clusters (Figs. 22 and 23). The hyperchromatic nuclei often are distinctly vacuolated (Fig. 22). The nucleoli number from one to several, and vary considerably in size and shape; many are irregular, and distinctly acidophilic. Here and there are small patches of necrosis. In some nearby cells, and less often elsewhere, the cytoplasm is wholly or partly hyalinized and eosinophilic; the appearance is somewhat reminiscent of diseased liver cells with Councilman bodies. The larger tumors have very few collagen or reticulum fibers.

The blood vessels in most tumors are scanty; occasionally they are abundant.

It is difficult at present to classify these ne-

oplasms more precisely than as "mesenchymal tumors." Although superficially they resemble fibromas or, when more invasive, fibrosarcomas, the paucity of fiber formation, and the presence of relatively numerous giant cells in the larger growths lead us to doubt a purely fibroblastic origin. Similarly, the lack of the characteristic pattern usually exhibited by nerve sheath tumors makes a Schwann cell origin questionable. Mesenchyme has so great a capacity to differentiate into diverse cell types that further studies are required before the histogenesis of the goldfish tumors can more accurately be determined.[1]

Occurrence of the neoplastic disease in certain pools; probable absence of hereditary factors in its development.—Twenty-eight of the 30 tumor-bearing goldfish of this series came from 3 small concrete outdoor pools.

The first pool had been maintained for a number of years in the court of an Atlantic City hotel. It measured 12 feet in diameter and 2 feet in depth. The original stock of goldfish came from a large hatchery in which no tumor-bearing fish had ever been found. Since the fish failed to breed in the pool, new individuals from the same hatchery were added from time to time in order to maintain an average number of 20. It is very unlikely that any of the fish in this pool were siblings. During the summer an abundance of aquatic plants grew in the soil of wooden boxes sunk beneath the surface. Once a year the pool was drained and cleaned but the wooden boxes and soil were left intact. In 1938 one of us noted that several fish had tumors. Similar growths had been seen previously by the caretaker, who had systematically removed such afflicted fish and replaced them with healthy individuals. During the next 3½ years the stock was carefully examined by us at intervals of 5 to 10 months and all tumor-bearing fish were removed to our laboratory. At each inspection 2 or more fish with new tumors were found, a total of 17 being collected during the entire period. The tumors were always fairly uniform in size, with a mean diameter of 8 mm., indicating considerable uniformity in their development during the intervals between removal of tumor-bearing fish.

The second pool is located in the garden of a private residence in Atlantic City. It is smaller than the first and contained fewer plants. It had been stocked with goldfish from undetermined sources 13 years prior to our first examination in 1942. The population had slowly dwindled, without breeding, until only 10 fish remained. Each winter the pool had been drained and the fish transferred to indoor tanks. Two of the 10 fish were found to have multiple tumors (Fig. 4). The owner stated that a number of fish had died during the preceding few years with similar growths.

The third pool is in the Philadelphia Zoological Garden. It is larger than the first and contains many aquatic plants. A fairly constant population of approximately 55 goldfish, obtained from many diverse sources, had been maintained in it for 5 years prior to our first examination in 1946. Fantail and swordtail varieties as well as common goldfish were present. Each winter the pool was drained and the fish transferred to indoor tanks. Beginning in 1946 all fish were carefully examined at intervals. On the first examination 7 of a population of 55 fish were found to have typical tumors. The tumor-bearing fish were allowed to remain in the pool. Within a year, 2 other fish had developed neoplastic growths, making a total of 9, or an incidence of 16 per cent. The subsequent course of these tumors will be discussed presently.

In the 3 pools the heterogeneous composition of the population is strong evidence that hereditary factors played no part in the development of the tumors. On the other hand, there were no obvious features noted by us that could be regarded as tumor-inducing.

In contrast to the high incidence of the neoplastic disease in these pools is its uncommon occurrence in other pools. Among approximately 200 goldfish in the pond of the University of Pennsylvania Botanical Garden only two individuals with neoplasms were noted during several years' observation. In the ponds of a State Fish Hatchery near Philadelphia only one fish with tumor was found among 70 examined; the experienced and cooperative superintendent of this hatchery regarded the neoplastic disease as rare. In a large commercial goldfish hatchery, also located near Philadelphia, the disease was apparently unknown. However, the owner of still another hatchery near Lancaster, Pennsylvania, recently informed us that every year he culled out about 25 tumor-bearing fish from a population of many thousands. The removal of diseased fish before sale to dealers perhaps accounts for the fact that the disease is very seldom seen in the stock of retail stores. None of over 500 individuals purchased by us from dealers in Phil-

[1] We wish to thank Dr. Purdy Stout of Columbia University for examining sections of some of our tumors. He also doubts a fibroblastic or Schwann cell origin of the neoplasms. A recent paper of Dr. Stout's gives an excellent discussion of the complexity of mesenchymal tumors (19).

TABLE II: RATE OF GROWTH OF TUMORS

This table summarizes records of observations on 10 tumor-bearing fish which were kept from 12 to 40 weeks. Sizes at the beginning and end of observations are given in millimeters and refer to the two greatest horizontal diameters; vertical diameters are omitted because of the difficulty of measuring accurately the depths of the tumors.

Designation of tumor	Duration of observation, (weeks)	Course
4	27	Solitary tumor on left side near level of dorsal fin, 7 × 5 mm.; no definite change in size (Fig. 2).
5	40	Solitary tumor on left side near posterior end of dorsal fin, 5 × 5 mm.; slowly increased to mass measuring 10 × 10 mm.
6	12	Large solitary tumor on left side; approximately 10 × 10 mm. when first noted; grew very rapidly to mass measuring 35 × 31 (Fig. 1).
7	24	Large solitary tumor on top of head, 15 × 10; during first several months of observation slow growth; thereafter rapid growth (Fig. 6).
8	40	Solitary tumor on tail, 7 × 3; slow increase to 12 × 5.
9	40	Solitary tumor on right operculum, 10 × 10; no definite change in size.
10	14	Solitary tumor on upper border of the sclerocorneal junction of the left eye, 4 × 4; no definite change in size.
11	14	Solitary tumor, directly above the right eye, 10 × 4; no definite change in size.
12	16	Solitary tumor at posterior margin of right operculum, 15 × 4; no definite change in size.
13	16	Solitary tumor on caudal fin, 20 × 10 mm. (Fig. 3); slow increase to approximately 30 × 20 mm.

adelphia and observed in our laboratory for periods of over 6 months has developed tumors.

Geographic source, age, race, and sex.—All but two of the fish in our series came from pools located in Pennsylvania or New Jersey. The other two were obtained from Maryland and from California.

Little information is available concerning age. The fish shown in Fig. 1 was approximately 5 years old; that shown in Figs. 20 to 25 had lived in a pool for about 15 years; the one in Figs. 4 and 5, for about 13 years. What fractions of the life-span these ages represent is not known. No tumors were found in small and presumably young fish.

Factors of sex and of race of goldfish appear to play no part in the etiology of the neoplastic condition.

Course of the tumors.—In Table II are summarized the records of observation on the rate of growth of tumors in 10 fish which were studied for periods of from 12 to 40 weeks. Five of the tumors did not change appreciably in size or appearance. Three increased slowly to masses having approximately twice the original diameters. Two tumors grew rapidly. One of these (shown in Fig. 6) after slow increase during the first 6 months of observation, became more active and attained large size. The most rapidly proliferating tumor of the series was approximately 10 × 10 mm. in size when first noted, and grew to a mass of 35 × 31 mm. within 12 weeks (Figs. 1).

The observations described above concern a group of tumors that had passed the initial stages of the neoplastic process; *i.e.*, that had become established and attained considerable size. We now come to another group, in which most of the tumors were small and in which we were able to observe their development and subsequent fate. These studies were made on the goldfish in the pool of the Philadelphia Zoological Garden. Each tumor was carefully measured at intervals during a period of 21 months; in the first year the examinations were spaced approximately 3 months apart. The measurements of the tumors were accurate to within 1 mm. The observations are summarized in Table III. It will be seen that some of the tumors gradually disappeared, while new tumors developed. For example, in September, 1946, goldfish 46-1 had a solitary tumor, measuring 3 mm. in greatest diameter, on its left operculum. After remaining static for about 9 months this growth slowly disappeared; it did not recur during the following year. But by March, 1947, a new tumor had arisen, located on the left pectoral fin; it persisted for about 6 months and then disappeared, so that in June, 1948, the fish had no tumors at all. A more striking example of regression and new appearance of tumors is to be had in goldfish 46-3 in which 14 different areas were at one time or the other affected with neoplasms; all of these tumors ultimately disappeared. In general, the tumors which dwindled away were relatively small. On the other hand, the larger tumors, those in goldfish 46-2, 46-4, and 46-7, persisted, two without significant change, one increasing slowly.

It seems reasonable to conclude that the goldfish tumors pass through sequential phases: through initial stages during which they are unstable and often regress, through a more stable phase of persistence (even though they may remain static for long periods); finally, an active phase of growth,

TABLE III: COURSE AND FATE OF TUMORS

Designation of Goldfish	Location	Date of examination (largest diameter in millimeters) Sept. 14, '46	Dec. 18, '46	Mar. 12, '47	June 11, '47	Aug. 27, '47	June 22, '48
GF 46–1	L. operculum	3	4	3	3	0	0
	L. pectoral fin	0	0	2	1	1.5	0
GF 46–2	R. side	6	8	8	9	8 (Removed by biopsy)	
	L. pectoral fin	14	11	15	16	14 (" " ")	
GF 46–3	R. side	5	6	7	7	6	0
(Fan-	" "	?	3	5	5	3	0
tail)	" "	?	2	3	3	2	0
	" "	?	3	3	4	4	0
	" "	?	3	2	3	3	0
	" "	0	0	0	3	0	0
	" "	0	0	0	3	0	0
	L. side	2	4	3	3	2	0
	" "	0	0	3	3	3	0
	" "	0	0	0	2	0	0
	Tail	1	1	0	0	0	0
	"	1	1	0	0	0	0
	"	1	1	0	0	0	0
	"	0	1	0	0	0	0
GF 46–4 (Fan-tail)	Base of dorsal fin	10	10	12	(Fish not located)		
GF 46–5	R. side	5	4	4	0	3	3
(Fan-	L. side	0	0	2	0	0	0
tail)	" "	0	0	2	0	0	0
	R. eye	0	0	0	0	0	7
	L. eye	0	0	0	0	0	7
GF46–6	Dorsal fin	1	0	0	0	(Fish not located)	
GF 46–7	R. eye	10	10	10	10	11	11
GF 47–8	R. side	0	0	3	3	2.5	4
(Sword-	L. side	0	0	0	3	0	0
tail)							
GF 47–9	Anal fin	0	0	5	8	5	0

with invasive properties, may be attained. The factors responsible for any of these phases are unknown.[2]

Exposure of goldfish to a presumably tumor-inducing environment.—It seemed probable that each of the 3 pools described above provided an environment peculiarly suited for induction of the neoplastic process. Accordingly we attempted to find out whether fish from a "clean" source would develop tumors if placed in one of these pools. In June, 1947, 31 large and normal-appearing goldfish were placed in the pool at the Zoological Garden, the stock of which was temporarily moved elsewhere. Four months later when the fish were transferred to indoor tanks for the winter, none had developed any sign of tumor. In June, 1948, the upper part of the cornea and sclera of one eye of a single individual became considerably thickened, the appearance resembling one of the sclero-corneal tumors illustrated in Figs. 4, 5 and 13. None of the other fish, one year after the experiment began, showed evidence of tumor. This experiment, which must be considered as purely exploratory, indicates that if certain environments contain tumor-exciting factors for goldfish, such factors are either weak or very slow in their action.

Transplantation experiments.—In the studies here reported healthy looking portions from 6 tumors were transplanted into 99 fish. Most of the inoculations were made into the anterior chamber of the left eye; in addition, fragments of some of the tumors were also introduced into the corium or subcutaneous tissues. Either common or "telescope eye" goldfish served as experimental animals; the latter were used occasionally because of the greater depth of the anterior chamber in their eyes. The recipients were wrapped in wet cotton to the level of the eyes. The operative procedures were similar to those successfully used by us in transplanting frog carcinoma, and epithelioma of catfish (8, 9). Briefly, an incision was made in the superior region of the sclero-corneal junction; through this wound a bit of tumor tissue was introduced into the anterior chamber with a finely pointed forceps. Unlike the fluid aqueous humor of terrestrial animals, that of fishes is quite viscid and if care is not exercised, will carry the implant along as it exudes from the wound.

The results of these experiments are summarized

[2]For a discussion of developmental stages in neoplasia the recent papers by Berenblum (1), and by Rous and his associates (10, 15) should be consulted.

in Table IV. In no case was any growth noted in a homotransplant.

Autotransplantation to the anterior chamber of each eye was carried out in 4 fish and progressive growth occurred in one of them (goldfish 7). The course of the primary tumor and of its autotransplants reveal an interesting relationship which will now be detailed.

The primary tumor was located on the top of the head just posterior to the eyes. When first examined, on October 14, 1940, it measured 1.5 × 1.0 cm. Transplants of the tumor made to the anterior chamber of the eyes of 22 goldfish at this time failed to grow and soon regressed (7a, Table IV). During the following several months the tumor grew very slowly. Transplantation was again attempted (December 10, 1940) without success (7b, Table IV). On January 4, 1941, autotransplants were made to the anterior chamber of both eyes and into the subcutaneous tissue of the trunk (7c, Table IV). The latter regressed, but the intraocular implants in both eyes persisted, though at first their increase in size was slow. In the middle of March, however, the primary tumor began to grow rapidly, and concomitantly the transplants in both eyes grew vigorously (Figs. 24 to 29). The growth of the primary tumor and of its autotransplants continued at comparable rates for 9 weeks. Renewed attempts to transfer bits of either the primary tumor or one of its autotransplants to the eyes of other goldfish were again unsuccessful (7d and e, Table IV), and the fish died soon afterward. At autopsy the primary tumor was found to have extended to the bone, which was, however, not invaded. There were no metastases in the internal organs. This experiment raises the question whether host factors which stimulate growth of a primary tumor act also upon intraocular autotransplants. A discussion of this interrelation may be found in a recent paper by Greene (5).

In the experiments described above, the tumor tissue used for transplantation consisted of bits about 1 to 2 mm. in diameter. In a more recent experiment a suspension of tumor cells was used (from goldfish 46-2) and Sudan III was injected simultaneously in the hope of stimulating cell proliferation. Briefly, the procedures were as follows: In August, 1947, two tumors, one measuring 14 × 8, the other 8 × 8, were removed and cut into fragments which were gently massaged through a 40-mesh stainless steel sieve into frog Ringer's solution. The resulting thick suspensions contained many free cells and some larger particles. About 0.01 cc. was injected into the anterior chamber of the right eye of 27 normal goldfish under urethane anesthesia. In addition every fish also received 0.05 cc. subcutaneously in 2 locations on the right side of the body near the dorsal fin. Directly following the introduction of the tumor suspension, 0.05 cc. of saturated solution of Sudan III (Grübler) in olive oil was injected into one of the locations. As control, a suspension of muscle and skin from a normal goldfish was prepared in the same way as the tumor and was injected into corresponding locations on the left side; Sudan III was also injected into one site.

Twenty of the fish survived for more than 10 months. None showed any evidence of tumor. The transplants in the anterior chamber did not grow and all traces of them disappeared within 6 months.

It is pertinent to state here that attempts at

TABLE IV: SUMMARY OF TRANSPLANTATION EXPERIMENTS

Designation of tumor	Number and kind of recipients	Site of inoculation	Result
4	1 telescope eye goldfish	Ant. chamber	No growth, complete regression
	3 Sunfish	" "	" " " "
	12 common goldfish	" "	" " " "
7a	11 Telescope eye goldfish	" "	" " " "
	11 common goldfish	" "	" " " "
7b	4 common goldfish	" "	" " " "
	8 common goldfish	Caudal fin	" " " "
7c	Autotransplant	Ant. chamber of both eyes	Progressive growth
		Subcutaneous	No growth, complete regression
7d	4 Telescope eye goldfish	Ant. chamber	" " " "
	6 common goldfish	" "	" " " "
7e (Transplant from left eye)	8 common goldfish	" "	" " " "
12	Autotransplant	Ant. chamber of each eye	" " " "
13	Autotransplant	Ant. chamber of each eye	" " " "
14	Autotransplant	Ant. chamber of each eye	" " " "
46–2	27 common goldfish	Ant. chamber	" " " "
		Subcutaneous	" " " "

transplanting tumors of any species of fish have been almost uniformly unsuccessful. The only exception is the epithelioma of the lip in catfish which we have successfully transplanted in 25 instances to the anterior chamber of the eye and between the layers of the cornea (9). Previous attempts to transplant goldfish tumors of the kind dealt with in this paper have been made by two groups of investigators. Eguchi and Oota (4) inoculated bits of a "fibroma" growing on the back of a goldfish into other fish of the same species; no growth occurred. A "fibrosarcoma" of a fin in a goldfish was inoculated into the caudal fins of 3 other goldfish by Montpellier and Dieuzeide (11). After the lapse of a year no tumors had developed; 6 more fish inoculated in a later experiment likewise showed no growth. Failure to transplant the goldfish tumors is not surprising when one considers that in these experiments an inadequate number of recipients were used; that the recipients were probably not related genetically to the tumor-bearing fish, and that most of the tumors probably had not as yet reached a stage of fully developed malignancy. Reviewing our own experiments, the primary tumors of goldfish numbers 4, 12, 13, 14, and 46-2 were growing rather slowly, showed only slight invasive properties, and had produced no metastases. Fully developed malignancy of the cells had therefore not been attained and hence none of the transplants became established. In goldfish number 7, however, the tumor became active and its autotransplants did become established. In this case, when the primary tumor began to grow vigorously its intraocular transplants also grew progressively.

SUMMARY

1. Neoplasms of mesenchymal origin occur commonly in the corium of the goldfish, *Carassius auratus*. Studies on 30 tumor-bearing goldfish are reported in the present paper. The tumors are usually solitary, less often multiple. While the rate of growth is slow in most tumors, some grow very rapidly and attain relatively great size; in one individual, one-third the size of the entire body. The larger tumors are infiltrative, the smaller are circumscribed, though not encapsulated. Metastasis has not been observed. Microscopically, the tumors resemble fibromas or fibrosarcomas, but the paucity of fiber formation makes a purely fibroblastic origin doubtful. It is also unlikely that the growths are of Schwann cell origin. The precise histogenesis of these neoplasms is as yet undetermined.

2. Prolonged observation of a number of fish bearing early tumors has shown that some of the growths may regress, while new tumors appear elsewhere. These observations support the hypothesis that neoplastic growth proceeds through several stages, and that in the earlier stages of the sequence the process may be reversible.

3. It is characteristic of the neoplastic disease that it occurs in certain pools and is absent or rare in others. There is indirect evidence that the tumor-inducing factors are either transmissible or environmental. Hereditary factors very probably play no part in the etiology of the disease. Exposure of healthy goldfish to a presumably tumor-inducing environment has led to the tentative conclusion that the carcinogenic factors are either weak or very slow in their action.

4. Attempts to transplant the tumor by inoculation of living tumor cells subcutaneously, or to obtain growth of homologous transplants in the anterior chamber of the eye were unsuccessful.

5. Autotransplantation in one instance, however, yielded vigorous and prolonged growth in the anterior chamber. The rate of growth of the transplant closely paralleled the rate of growth of the primary tumor, thus suggesting the existence of host factors which operate on both primary tumor and on its intraocular autotransplants.

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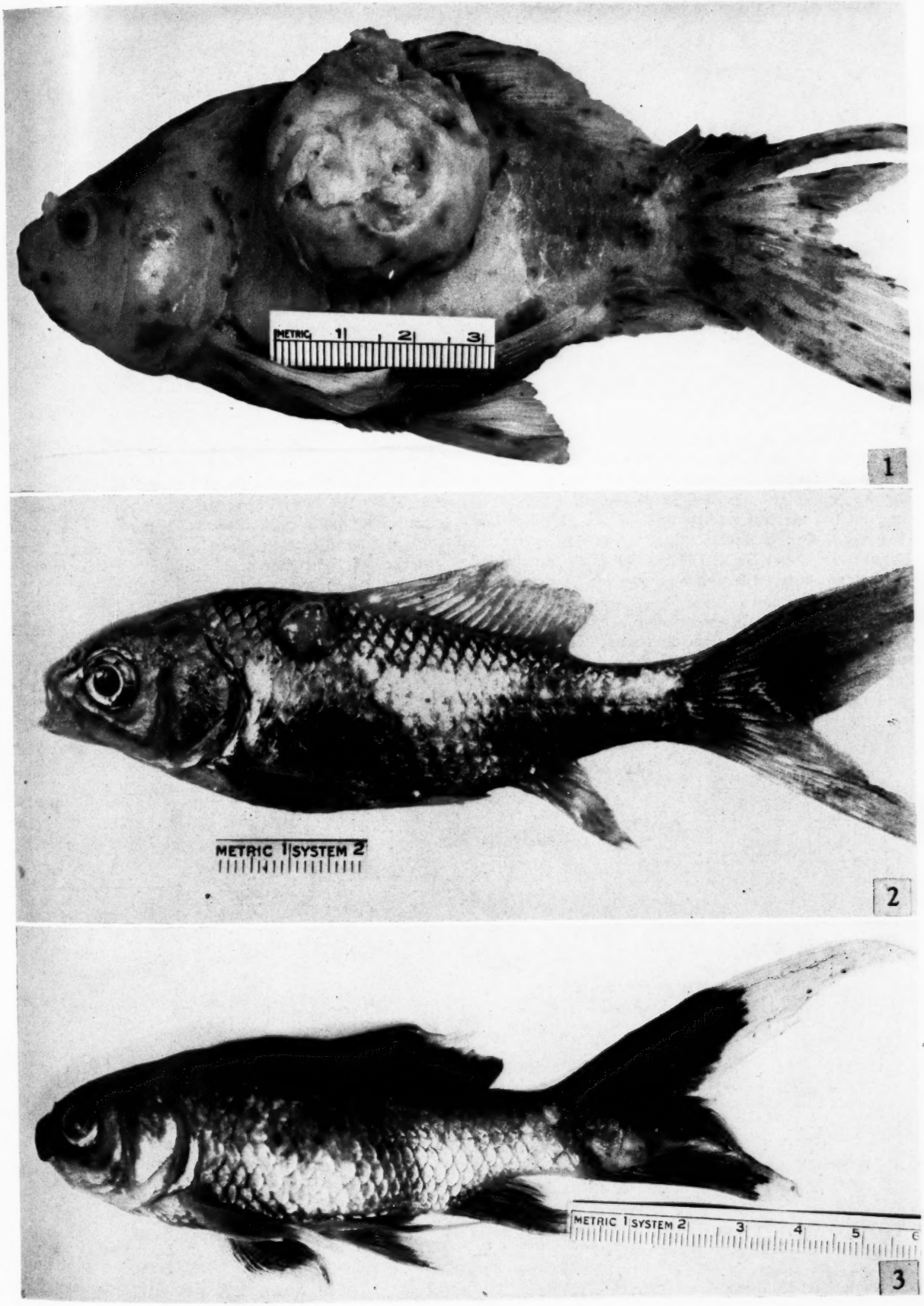
DESCRIPTION OF FIGURES 1 TO 3

Fig. 1 (tumor 6).—A solitary large tumor arising in the corium of a "calico" goldfish, about 5 years of age, and measuring in its overall length 14 cm., and from snout to beginning of tail 10 cm. The tumor is located on the left side of the body; it is hemispherical in shape, and projects 16 mm. above the skin the scales of which have been destroyed; the surface is generally smooth, but a few areas have become eroded. The growth measures 35 × 31 mm. in its greatest diameters, *i.e.*, it is approximately one-third the size of the entire body.

Fig. 2 (tumor 4).—A solitary tumor in a "fan-tail" goldfish. The tumor is located on the left side of the body, near the beginning of the dorsal fin; it has hemispherical shape, a smooth surface, and measures 7 × 5 × 5 mm.

Fig. 3 (tumor 13).—A solitary tumor on the caudal fin of a "fan-tail" goldfish. The growth is elongated in shape; it lies in a direction parallel to the fin rays, and measures 20 × 10 × 5 mm.



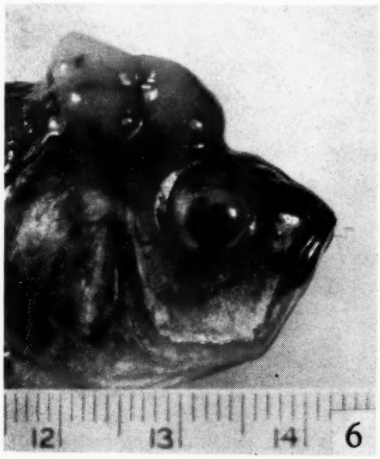
FIGS. 1-3

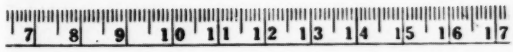
DESCRIPTION OF FIGURES 4 TO 6

FIG. 4 (tumor 20).—Multiple tumors in a goldfish, which has an overall length of 25 cm. and which measures 20 cm. from snout to beginning of tail. The largest tumor, 34 × 32 × 10 mm. in size, is deeply ulcerated. It is located on the left side, at the level of the posterior half of the dorsal fin. A second tumor, 14 × 10 × 3 mm., is also ulcerated, and lies at the margin of the operculum. A third tumor covers the upper portion of the cornea and encircles the eye.

FIG. 5 (tumor 20).—An enlargement of the tumors located on the eye and on the operculum shown in the preceding photograph. Mag. × 5.

FIG. 6 (tumor 7).—A solitary tumor on top of the head of a common goldfish. Note the transplant in the left anterior chamber of the eye. For the first 6 months of observation, growth was slow. During this time attempts to transplant portions of the primary tumor to the anterior chamber and the caudal fin of 34 goldfish were unsuccessful (Table IV, 7a and 7b); autotransplants in the anterior chamber of both eyes, however, did not regress, though they remained inactive. The photograph shows the appearance of the tumor at a time when rapid growth was just beginning; concomitantly, the hitherto dormant autotransplants also grew rapidly (Figs. 24 to 29). Two other attempts were made to transplant the growths (primary tumor and portions of the autotransplants 7d and 7e); both were unsuccessful. Both the primary tumor and its autotransplants grew progressively for 9 weeks, after which time the fish died.





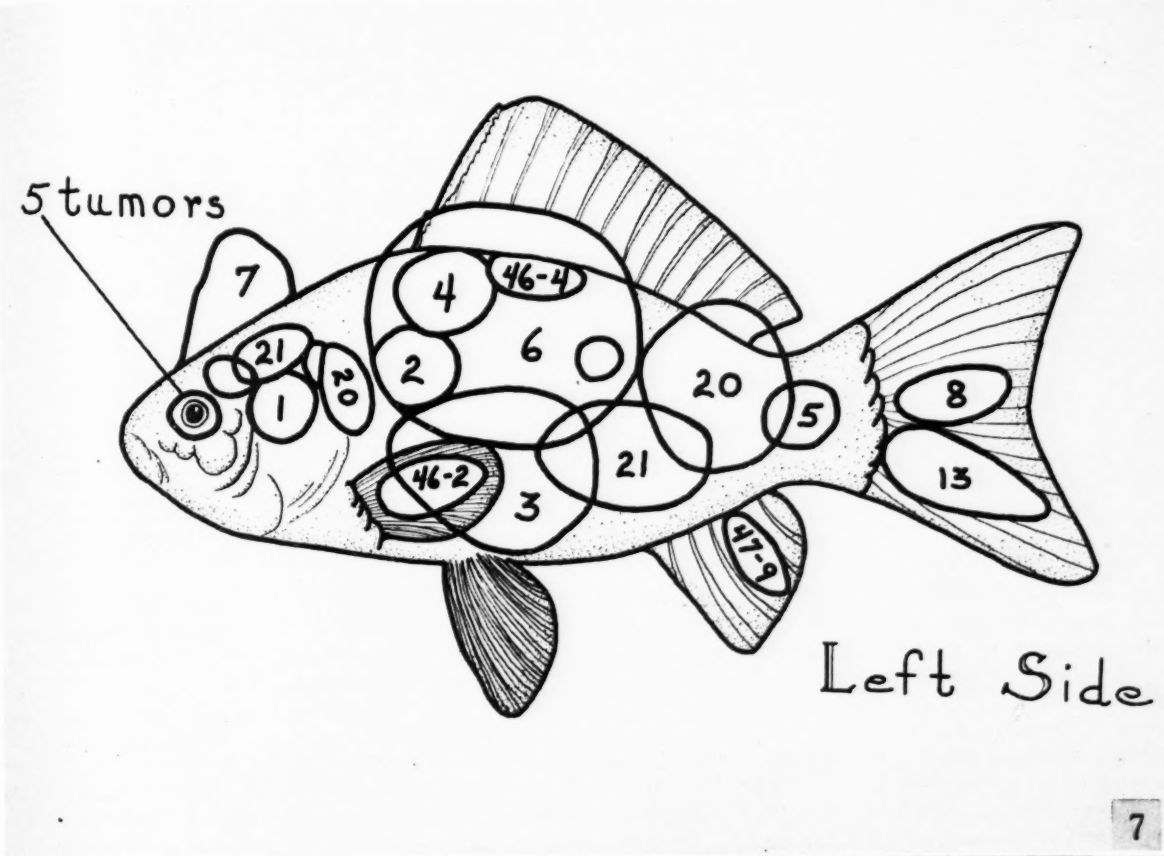
4

FIGS. 4-6

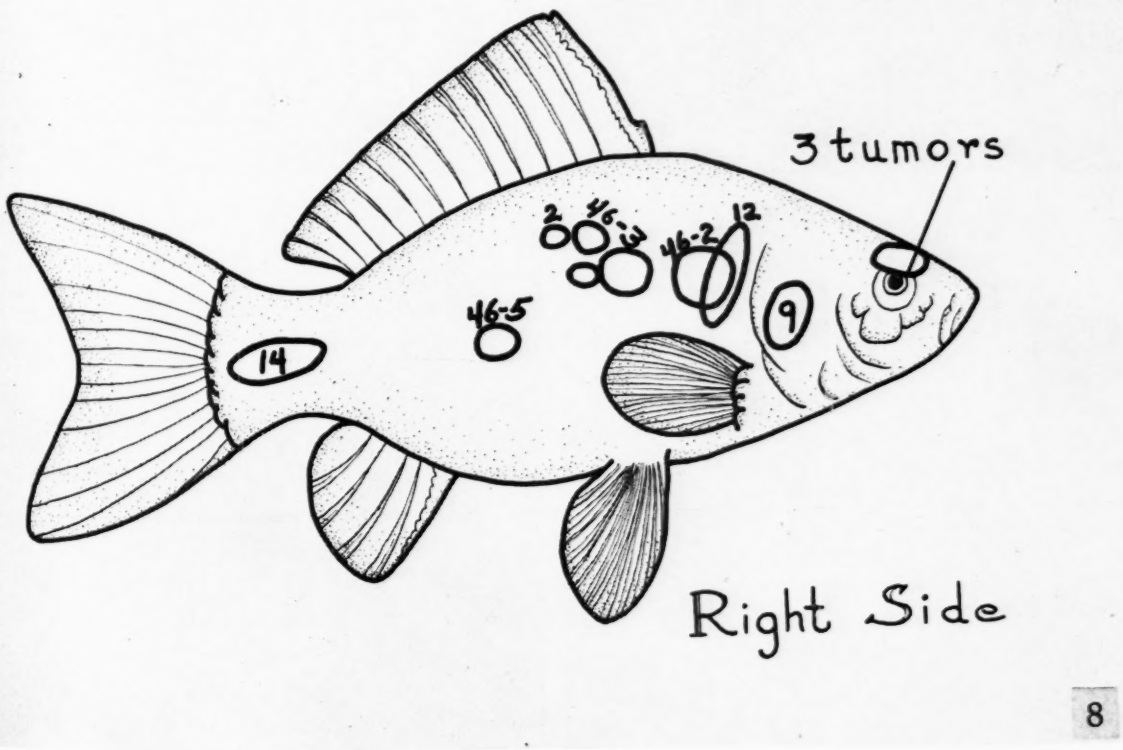
DESCRIPTION OF FIGURES 7 AND 8

Regional distribution of the tumors

The distribution graphs show that the neoplasms do not favor any particular location. The greater involvement of left over the right side of the body is regarded as fortuitous. The tumors are drawn to approximate scale. To avoid overcrowding of the graph we have omitted small tumors measuring less than 4 mm. in diameter and tumors numbers 15 to 19.


5 tumors
7
21
1
20
4
46-4
2
6
20
5
8
46-2
3
21
13
47-9
Left Side

7


3 tumors
2
46-12
46-2
12
46-5
9
14
Right Side

8

FIGS. 7-8

DESCRIPTION OF FIGURES 9 TO 13

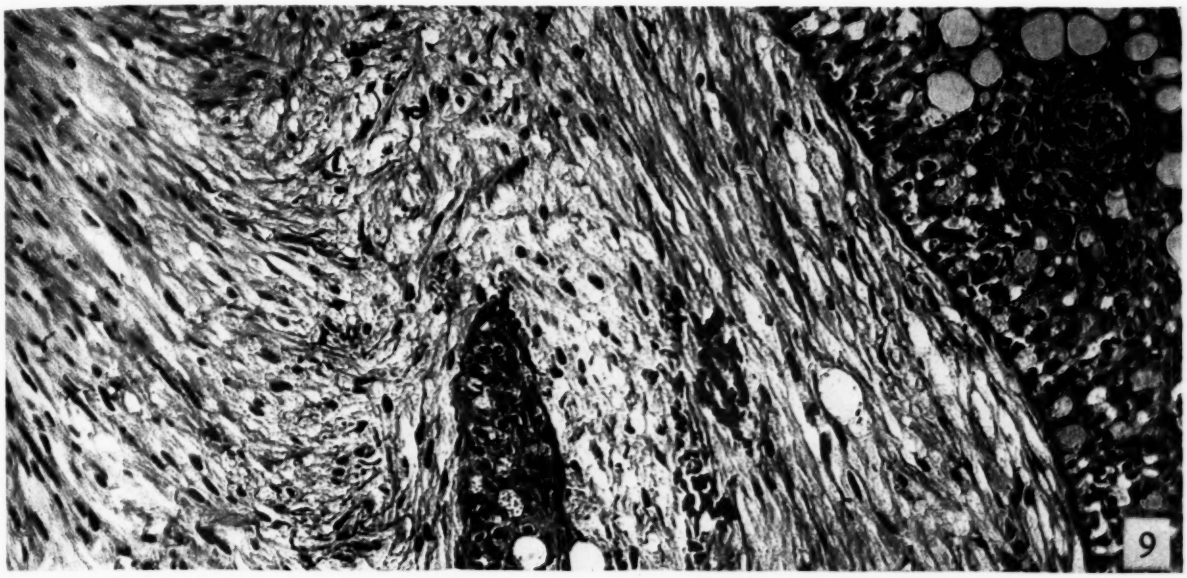
All sections were stained with hematoxylin and eosin.

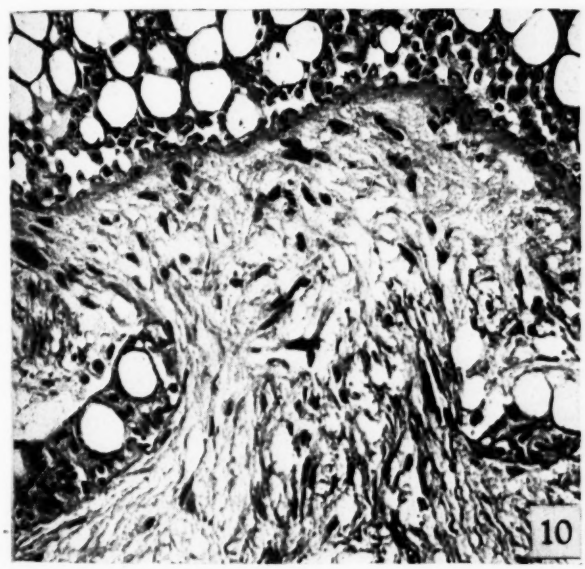
FIG. 9 (tumor 5).—This photograph shows the characteristic appearance of a small, early neoplasm arising in the corium. The surface of the tumor is covered by normal epiderm (shown in the upper right hand corner; a tongue of epiderm projects into the growth). The tumor has a loose structure, and is composed of elongated cells which tend to be arranged in fasciculi. The intercellular material is edematous and delicately fibrillar. Mag. × 200.

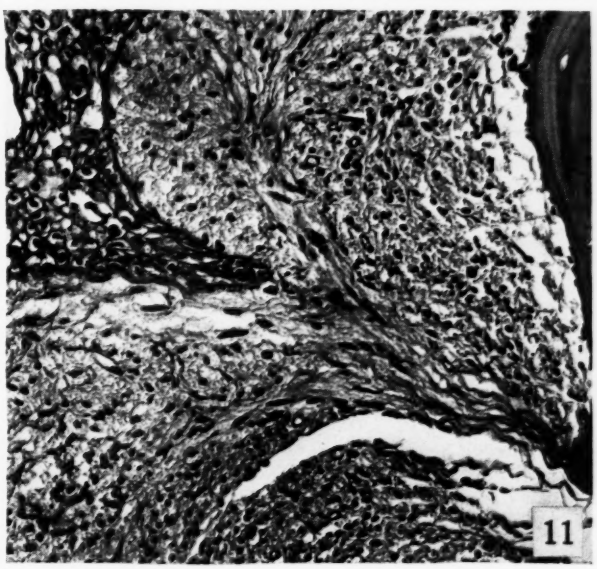
FIGS. 10 and 11 (tumor 5).—These photographs illustrate other areas from the tumor shown in Fig. 9. Mag. × 200.

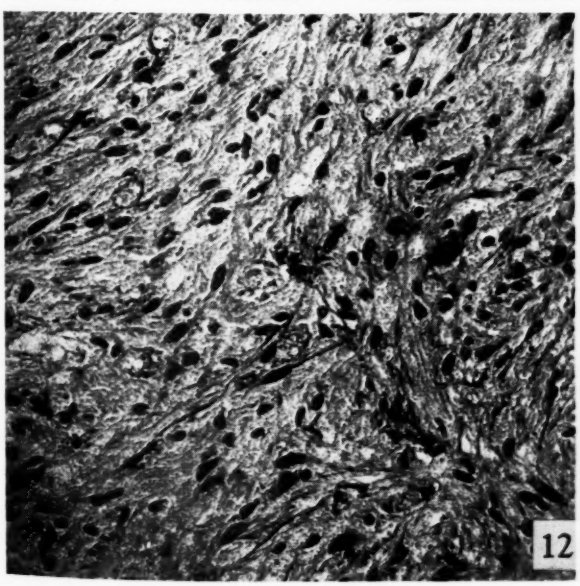
FIG. 12 (tumor 8).—The section is from a small tumor growing in the corium of the caudal fin. The component elongated cells are somewhat more closely packed than in the preceding illustrations. The nuclei have distinctly rounded rather than pointed ends. Mag. × 250.

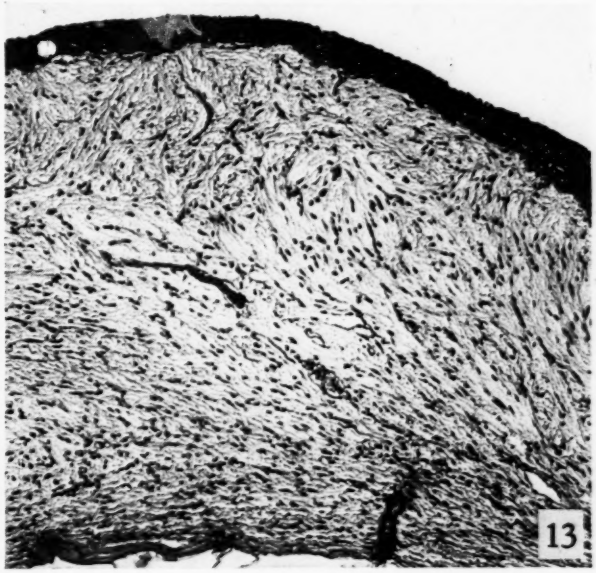
FIG. 13 (tumor 8).—Appearance of a corneo-scleral tumor such as is shown in Fig. 5. The surface is covered by conjunctiva. The growth has the same loose, fascicular structure as the tumors arising in the corium. In places there is a suggestion of a pallisading alignment of the nuclei. Mag. × 100.











FIGS. 9-13

DESCRIPTION OF FIGURES 14 TO 17

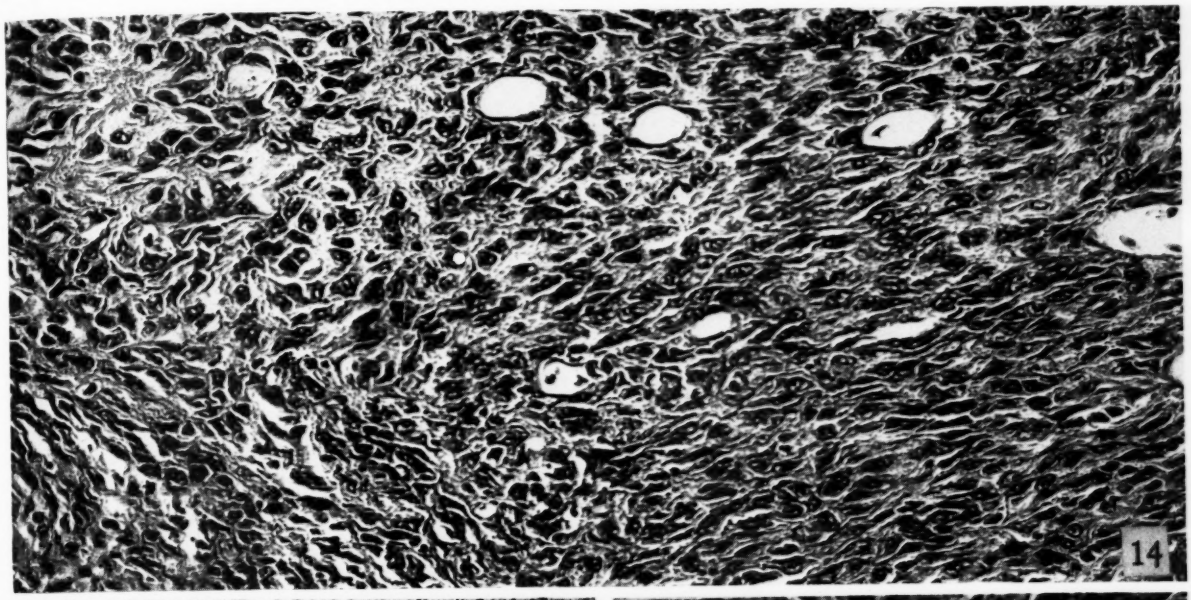
All sections were stained with hematoxylin and eosin

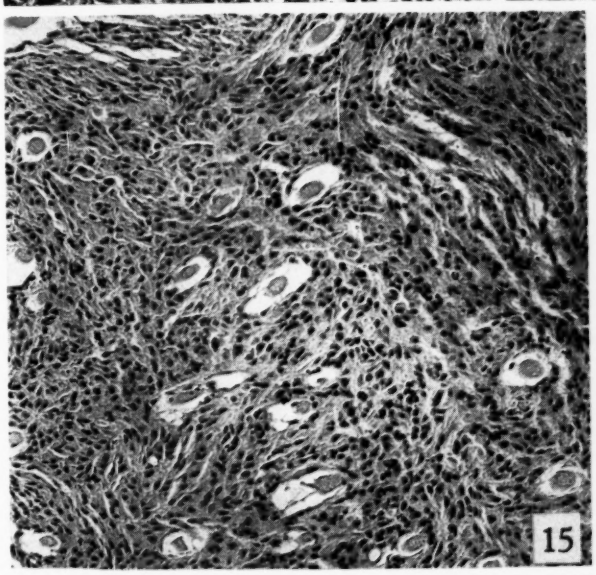
FIG. 14 (tumor 3).—Section from the very large tumor shown in Fig. 1. The component cells are more closely packed and less elongated than those of the smaller growths. Some cell groups are aligned in strands, while others have no particular arrangement. Note the prominence of the nuclei, in comparison with those shown in Figs. 9 to 11 at the same magnification. Mag. × 200.

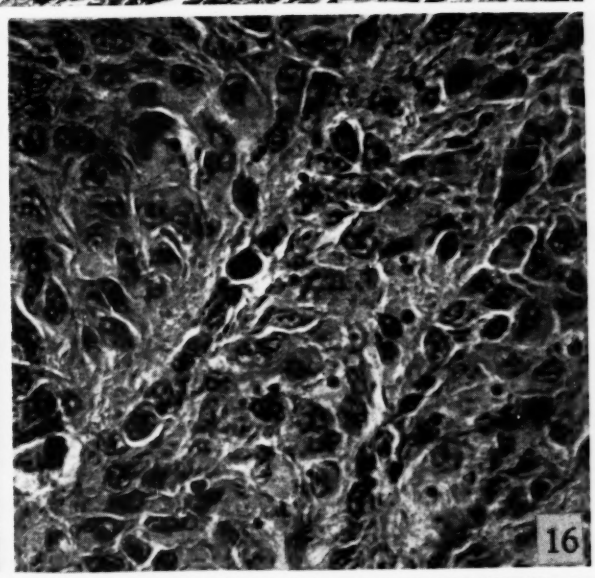
FIG. 15 (tumor 6).—Section from a large tumor which has infiltrated and destroyed the subjacent musculature. A few muscle cells still remain as pale atrophic bodies surrounded by an edematous zone. Mag. × 150.

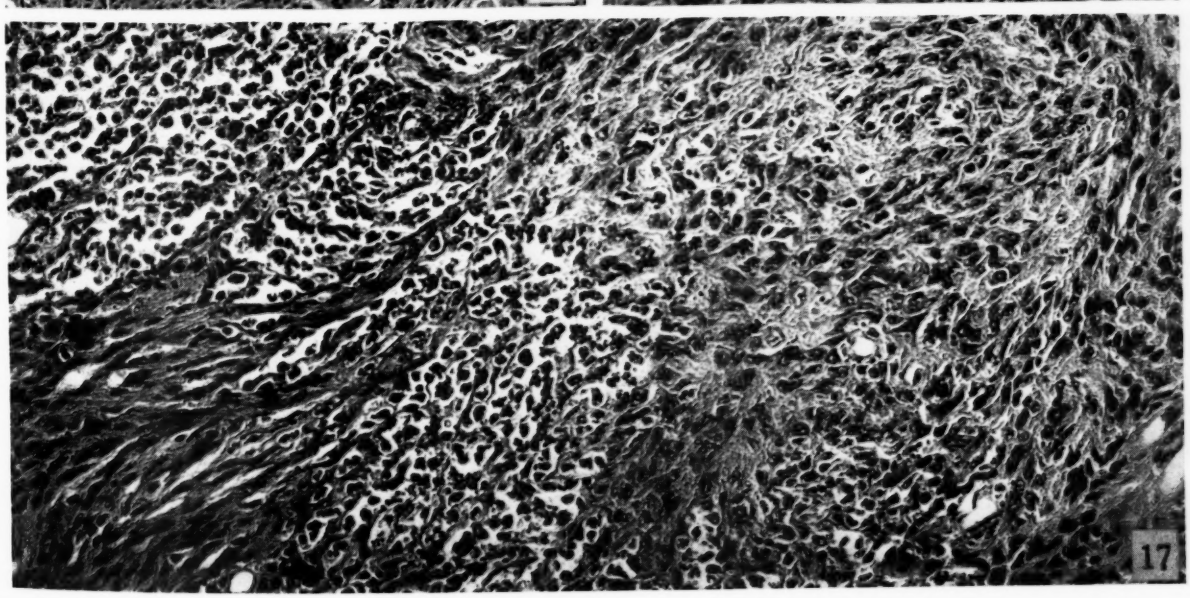
FIG. 16 (tumor 3).—Higher magnification of the cells shown in Fig. 14. The nuclei are prominent, and round rather than oval. There is very little intercellular substance. Mag. × 700.

FIG. 17 (tumor 3).—This photograph shows another section of the tumor illustrated in Fig. 14. Note that some cell groups are arranged as bundles which run at right angles to other fasciculi (left half of figure). Other cell groups have no particular pattern of arrangement (right half of photograph). Mag. × 200.









FIGS. 14-17

DESCRIPTION OF FIGURES 18 TO 23

All sections were stained with hematoxylin and eosin

FIG. 18 (tumor 7).—The appearance of nuclei in a representative field of the large tumor shown in Fig. 6. The nuclei vary in size, shape, staining properties and number of nucleoli. Mag. × 700.

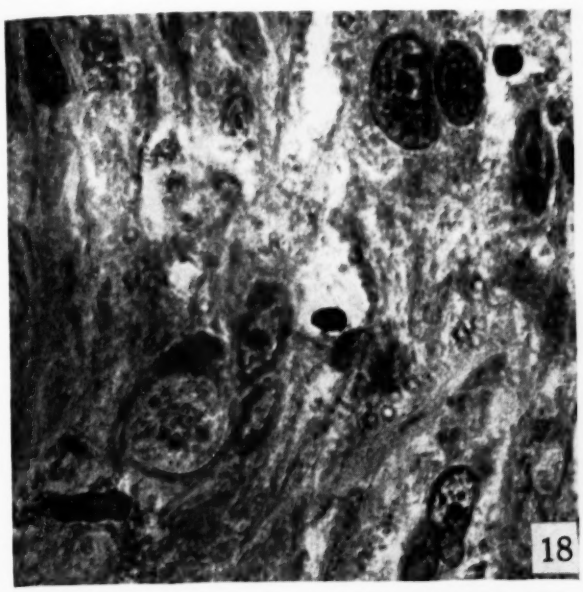
FIG. 19 (tumor 3).—Near the center of the photograph is a cell with a large hyperchromatic nucleus. Nuclei such as the one shown are common in large tumors. Mag. × 700.

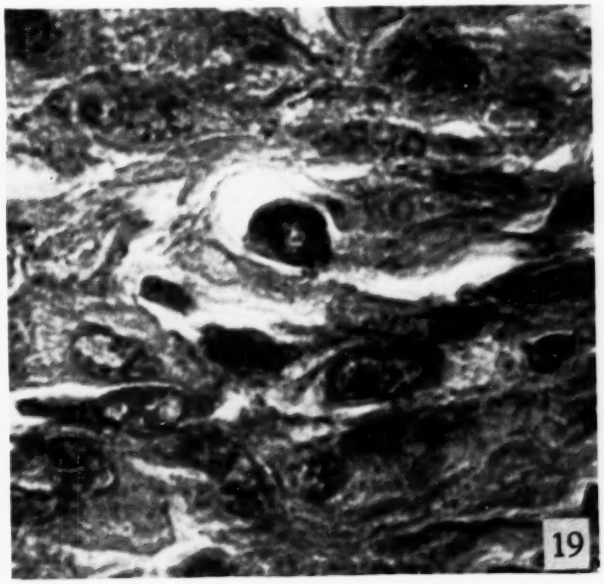
FIG. 20 (tumor 6).—The cells of this tumor, in the field shown, are elongated and have hyperchromatic nuclei. Contrast the appearance of these cells with those shown in Figs. 21 to 23 from different areas of the same large tumor. Mag. × 700.

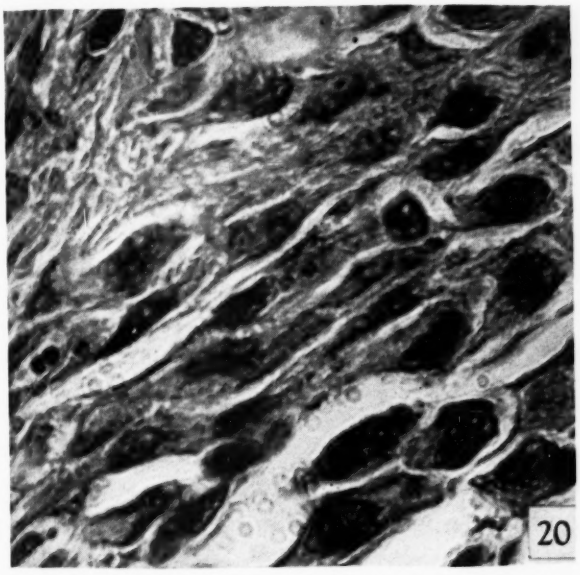
FIG. 21 (tumor 6).—The cells are pleomorphic. Close inspection will show that many of the large nuclei are vacuolated. Mag. × 700.

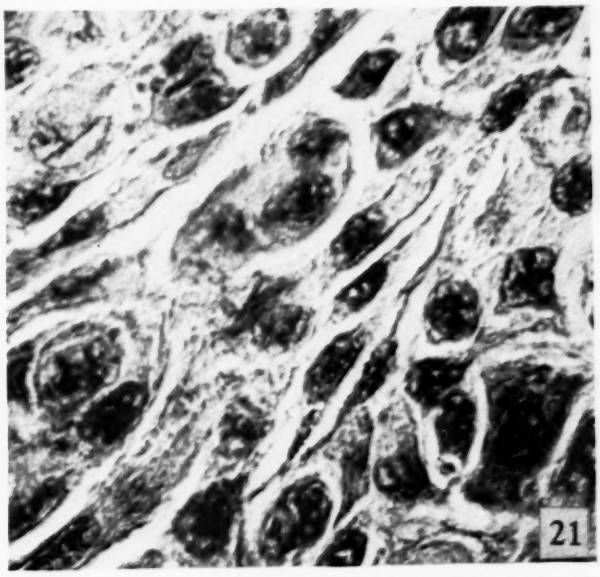
FIG. 22 (tumor 6).—Near the center of the photograph is an irregular shaped giant cell with deeply staining fused nuclei. Mag. × 700.

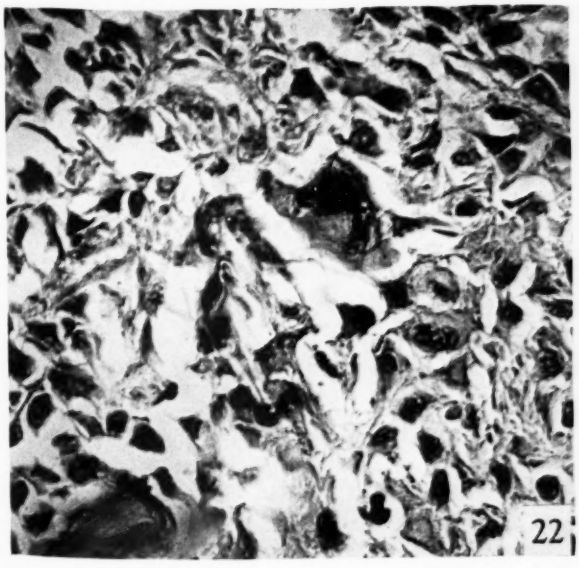
FIG. 23 (tumor 6).—A group of multinucleated giant cells is shown in the upper half of the photograph. Mag. × 700.

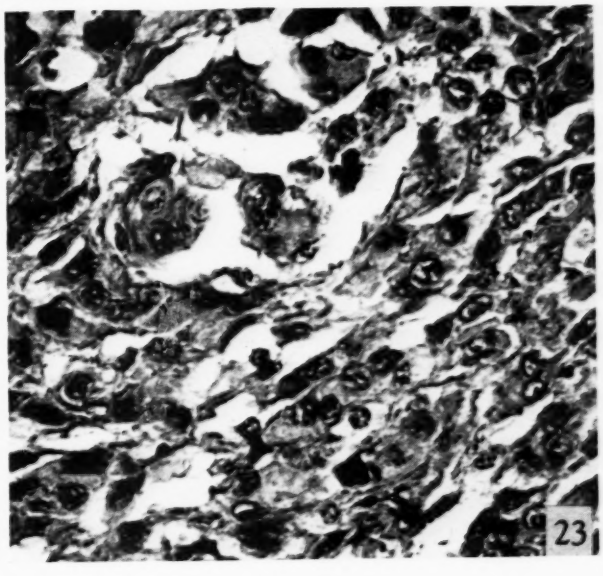








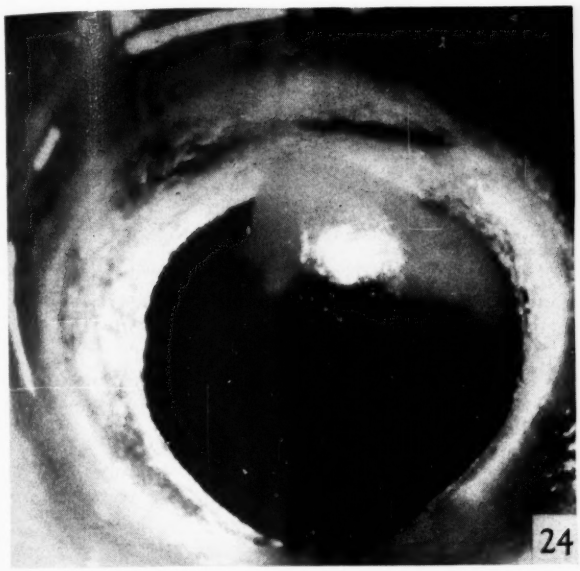


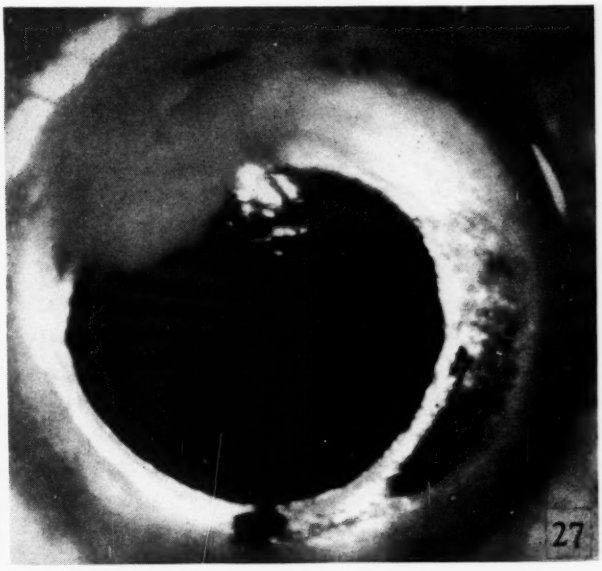


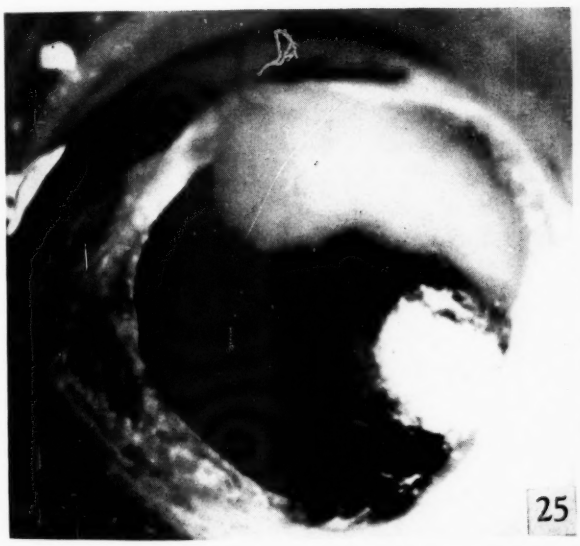
FIGS. 18-23

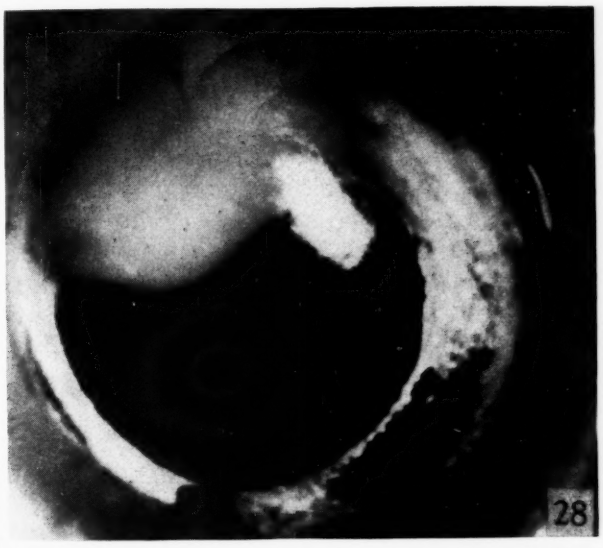
DESCRIPTION OF FIGURES 24 TO 29

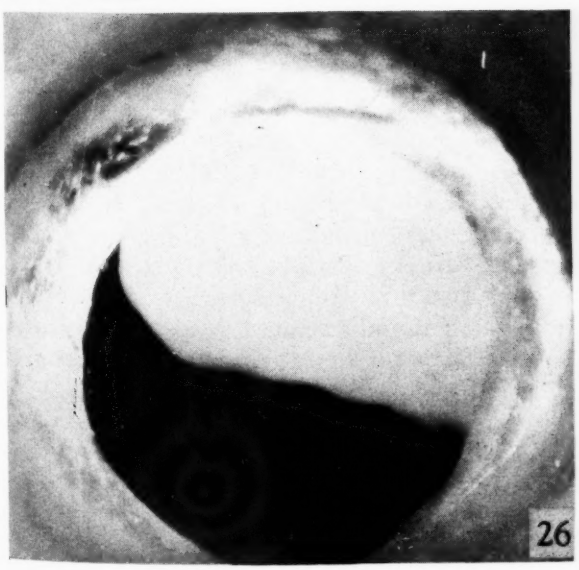
All illustrations are unretouched photographs of living tumors, *i.e.* autotransplants in the anterior chamber of the eyes. Figs. 24 to 26 show the appearance of the transplant in the right eye on March 15, April 12, and May 3, 1947, respectively. Figs. 27 to 29 illustrate the transplant in the left eye on the same dates. Note that the rate of growth became increasingly rapid. Mag. × 8.

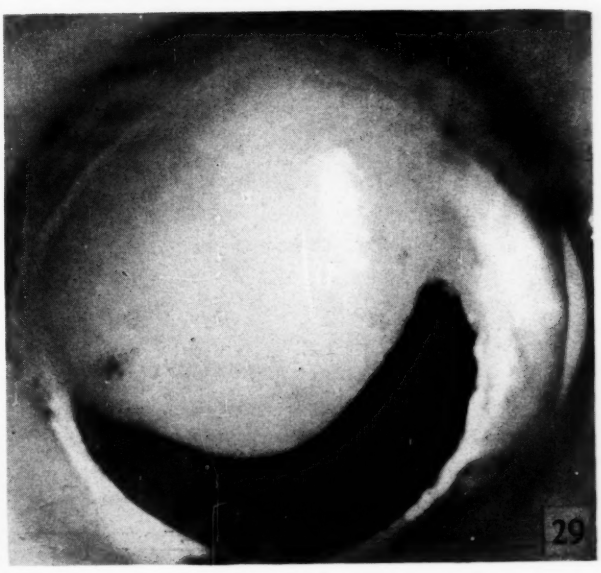












FIGS. 24-29

Studies *In Vitro* and *In Vivo* on the Effects of a *Staphylococcus Aureus* Extract on Mouse Carcinoma

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(Received for publication June 22, 1948)

Storage at low temperature of tumor cell suspensions in beef spleen extract (10) have been reported to delay and to inhibit the growth of dbrB adenocarcinoma in dba mice. The present report is an extension of this work to a similar study of the effect of a bacterial extract on tumor cell viability, as well as its effect on tumor cells *in vivo*.

The use of bacterial filtrates and metabolic products of bacterial growth in the treatment of a large number of tumors has been well reviewed and discussed by Shear (14) and by Dyer (6). For the most part, bacterial products in the treatment of tumors have not been effective. The material used in the present investigation differs from that described in most of the literature in that it is neither a bacterial filtrate, nor a bacterial metabolite, but is prepared from the actual organism itself. The only material reported closely resembling that used in these experiments is the one prepared from *Streptococcus pyogenes aureus* by Beebe and Tracy (2) in 1907 and found to produce some regression in lymphosarcoma in dogs.

The present report deals with (a) the effect of storage at low temperature on dbrB tumor in the presence of *Staphylococcus aureus* extract and (b) the effect of *Staphylococcus aureus* extract therapy on dbrB tumors in dba mice.

MATERIALS

Male hybrid dba mice approximately 6 to 8 weeks old and weighing between 15 and 20 gm. were obtained from the Jackson Memorial Laboratories. The dbrB adenocarcinoma was carried in dba mice received from the Institutum Divi Thomae, Cincinnati. Suspensions of the tumor injected into the mice produced tumors in every instance, and in no case was there evidence for a spontaneous regression, making the methods employed reliable criteria for studying the effect of storage and bacterial extract on the growth of the tumor.

The protein-free, water soluble extract of *Staphylococcus aureus* prepared as described by Nutini, Kelly and McDowell (13) was used throughout the experiments. The procedure consisted essentially of freezing and thawing 3 times in succession suspensions of the organisms in normal saline, precipitating the protein with alcohol and removing the latter by evaporation *in vacuo*. The extract was sterilized by passage through a Seitz filter. The strength of different batches of the material was compared on the basis of the dry weight per ml. The two lots of material employed in these experiments contained 284.3 and 309.3 mgm. per ml., dry weight.

PROCEDURES

Using aseptic technic the tumors were removed from stock dba mice for inoculation, the tumor pulp passed through a fine metal mesh, collected in a small graduated cylinder, and then diluted with twice its volume of Tyrode's solution. This suspension was then divided into 2 equal parts, to one of which was added Tyrode's solution in the proportion of 3:1, while to the second portion was added a similar volume of *Staphylococcus aureus* extract. The 2 suspensions aseptically placed in vaccine bottles were stored in the refrigerator at approximately 6° C.

One-tenth of a milliliter of the tumor suspension was inoculated into the dorsal shoulder region of the test animal, the site being selected because it is in such a position as to make it impossible for the animal to irritate the tumor by scratching or biting. One group of experimental and one of control animals were inoculated with the tumor suspensions immediately upon their preparation, the suspension being designated as "0 days' storage." Before injection of the stored suspensions into the animals the vials were allowed to come to room temperature.

One series of experiments was made in March of 1947, when the stored tumor suspensions were injected at intervals of 48 hours over a period of 8 days after the day of preparation. There were 27 control and 25 experimental animals in this series of 5 groups. In a second similar series of experiments, storage of the suspensions in the re-

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TABLE I: GROWTH OF DBRB ADENOCARCINOMA IN DBA MICE FOLLOWING STORAGE OF TUMOR SUSPENSIONS AT 6±2° C. IN TYRODE'S SOLUTION AND IN *Staphylococcus Aureus* EXTRACT

Storage period, days	No. mice and group	Takes and mortality,* %	Latent period,† average and range, days	Survival period,‡ average and range, days	Terminal volume of tumor, cu. cm.
			SERIES I, MARCH, 1947		
0	4 Control	100	14 (13–18)	15 (14–15)	10.35
	5 Exper.	100	14 (13–19)	12 (10–14)	7.68
2	6 Control	100	12 (11–14)	13 (8–14)	12.10
	6 Exper.	100	19 (11–26)	18 (13–28)	3.84
4	6 Control	100	12 (9–14)	13 (8–21)	3.78
	6 Exper.	34	20 (18–23)	17 (16–19)	7.56
6	6 Control	100	12 (10–14)	20 (13–24)	6.08
	4 Exper.	75	25 (18–31)	11 (6–17)	2.87
8	5 Control	100	12 (11–14)	19 (18–21)	17.34
	4 Exper.	50	21 (15–27)	15 (13–18)	3.74
			SERIES II, JULY, 1947		
0	6 Control	100	12 (11–16)	11 (6–13)	4.52
	6 Exper.	100	12 (11–16)	12 (9–21)	4.49
3	6 Control	100	15 (9–22)	13 (8–15)	1.98
	6 Exper.	50	23 (16–32)	25 (22–29)	4.51
6	6 Control	87	18 (13–26)	18 (10–23)	4.91
	6 Exper.	50	33 (22–45)	20 (8–36)	7.22
9	6 Control	66	24 (19–32)	16 (9–31)	6.81
	6 Exper.	0	..	..	..
12	6 Control	87	34 (26–53)	24 (4–53)	4.35
	6 Exper.	0	..	..	..
15	6 Control	16	29 (29)	19 (19)	59.32
	6 Exper.	0	..	..	..
18	6 Control	0	..	..	..
	6 Exper.	0	..	..	..
21	6 Control	0	..	..	..
	6 Exper.	0	..	..	..
24	6 Control	0	..	..	..
	6 Exper.	0	..	..	..

* Figures for percentage of takes and mortality are identical, as all mice developing tumors died of tumors.
† *Latent period* defined as time elapsing from inoculation with tumor cell suspension until tumor became palpable.
‡ *Survival period* reckoned as time elapsing from day tumor became palpable to date of death.

frigerator was continued for 24 days, with tests of the viability of the tissue being made at 72-hour intervals in a total of 54 control and 54 experimental animals.

In the second series of experiments the tumor was removed from 1 mouse in each group when the animal appeared to be near death, the tissue fixed in Bouin's solution and stained with hematoxylin and eosin.[1]

The latent period for tumor development was reckoned from the day of inoculation until the tumor became palpable; the survival time from the day the tumor became palpable to the day of death of the animal. Measurements of the tumors in 3 dimensions were made with calipers at 48 hour intervals and at the death of each animal.

In both of these experiments all animals developing tumors subsequently died thereof. In the July series of experiments a total of 69 mice failed to develop tumors in the control and experimental groups. Three months later 32 of these were inoculated in the groin with 0.1 ml. of a fresh suspension of dbrB adenocarcinoma. As the tumors became palpable in each of the animals they were treated daily with 50 mgm. dry weight of the *Staphylococcus aureus* extract in 0.4 to 0.5 ml., 8 animals receiving it intraperitoneally, 8 locally about the site of the tumor, 8 orally by means of a curved No. 22 needle, each animal receiving a total of 500 mgm. of the bacterial extract. Preliminary experiments with the extract had shown it to be non-toxic at even higher dosages than those employed here. The remaining 8 mice served as untreated control animals. The survival period for this third series of experiments was calculated from the last day of treatment until the death of the animal. Measurements in 2 dimensions were made of the tumors on the last day of therapy and again at the time of death of the animal.

RESULTS

In the March series of experiments (Table I) storage at 6° C. in Tyrode's solution for as long as 8 days resulted in 100 per cent tumor takes in the test animals with no significant alteration in the latent period and a slight prolongation of the

[1] We are indebted to Miss Loretta Langen for the preparation of these sections.

survival time. After storage of the tumor suspensions in *Staphylococcus aureus* extract, in the same series, the number of tumors developing decreased after the second day of storage, with a prolongation of the latent period but an apparent shortening of the survival time of animals receiving suspensions stored for 6 and 8 days. The tumor volume at the time of death of the animals was seemingly directly related to the length of the period of survival.

In the July series the control animals all developed tumors from suspensions stored for 3 days (Table I), the percentage takes thereafter decreasing to zero by the 18th day of storage. The latent period was comparable for unstored suspensions in the March and July series of experiments, but from the third day of storage upwards in the July series, it was prolonged in the control animals over that in the March series. The survival period for the 2 series was the same for the control animals. In the experimental animals of the July series, the number of mice developing tumors from suspensions stored in *Staphylococcus aureus* extract was reduced to 50 per cent after the third to the sixth days of storage, and to zero for all periods tested longer than this. The latent period was longer in this series, and the survival period may have been a bit longer, but the data are too few to be significant.

As compared with the experimental animals in the March series, both the latent and the survival period appeared to be somewhat prolonged in the July series.

The stained sections for both the control and the experimental groups for the entire series of experiments showed typical adenocarcinoma with numerous mitotic figures and apparently no difference in the histologic structure regardless of the length of storage prior to inoculation of the suspension, or whether stored in Tyrode's solution or in *Staphylococcus aureus* extract. Necrosis and hemorrhage were rarely encountered in these tumors.

In the third series of experiments (Table II) the administration of *Staphylococcus aureus* extract by the various routes in no way modified the ultimate fatal outcome of the experiment. Only local injection of the material appeared to decrease the rate of the tumor growth; whether this was a specific action of the extract or a non-specific response to local administration of a foreign material is not known. The survival period was not increased.

No immunity was conferred in surviving animals which had received tumor suspensions stored at 6° C. in Tyrode's solution for 18 to 24 days or in *Staphylococcus aureus* extract for 9 to 24 days, since all of these animals developed tumors upon re-inoculation with fresh tumor suspensions in Series III.

TABLE II: GROWTH OF DEVELOPING DBRB ADENOCARCINOMA IN DBA MICE DURING AND FOLLOWING 10 DAYS OF THERAPY WITH *Staphylococcus Aureus* EXTRACT ADMINISTERED BY DIFFERENT ROUTES

No. mice	Takes and mortality*, %	Latent period,† average and range, days	Survival period,‡ average and range, days	Average tumor area, after 10 days therapy, sq. cm.	Terminal average
			CONTROL		
8	100	9 (9–10)	8 (5–11)	2.52	4.80
			INTRAPERITONEAL INJECTION		
8	100	8 (8)	5 (3–8)	2.60	4.75
			LOCAL INJECTION ABOUT TUMOR		
8	100	8 (8–9)	8 (7–9)	1.10	1.95
			ORAL ADMINISTRATION		
6	100	11 (11–12)	9 (7–10)	2.70	3.15

* Figures for percentage of takes and mortality are identical.
† *Latent period* calculated from day of inoculation with tumor suspension.
‡ *Survival* calculated from day therapy discontinued. Therapy initiated on day tumor became palpable and continued for 10 days only.

DISCUSSION

The results of the storage experiments described above are in keeping with those of other workers (10–12). At low temperatures the metabolism of the individual cells is probably very low and since there is no proliferation the storage medium is adequate for a relatively long period of time. In the presence of the *Staphylococcus aureus* extract there is an inhibitory or killing effect on the stored tumor cells. A similar effect was produced by a crude liquor of *P. notatum* (12) by plant hormones (9) and by beef spleen extract (10).

In contrast to the results obtained with it in the storage experiments, the *Staphylococcus aureus* extract appeared to have little effect when used therapeutically. This is in agreement with the work of other investigators who have used various products of microbiological origin and found that

while *in vitro* there is a definite permanent damage to tumor cells or tissues, the same products used for the treatment of established tumors were for the most part ineffective (8, 3). Carr (3) for instance, found this to be the case with notatin and the Rous No. 1 sarcoma virus. There have been a few cases of regression of tumors due to therapy with bacterial products. Regression of tumors in mice was obtained by Shear (14) with meningococcus filtrates, and Duran-Reynals (5) reported regression of 2 mammary carcinomas in 52 mice treated with the washings of human typhoid bacilli grown on agar. Torrey and Kahn (16) obtained favorable results when *B. histolyticus* and *B. sporogenes* filtrates were directly injected into carcinomas in rats. Most workers, however, have found the same reaction as that reported here, namely that once the tumor is established the various bacterial substances thus far tested have no influence on the growth of the neoplasms, although Diller and Shear (4) have presented cytological evidence of damage to tumor cells when bacterial polysaccharides are used.

Much of the more recent work on the effect of bacterial extracts on various tumors has been on the hemorrhagic effects produced by the injected substances (1, 7, 15, 17). The materials used have caused severe hemorrhages in the established tumors, but in most cases have had no effect on their final development. The technic for determining the hemorrhage effect of the bacterial extract used in the experiments reported herein was not employed, but in only a few instances were hemorrhagic tumors observed, even when the *Staphylococcus aureus* extract was injected directly into the tumor.

SUMMARY

Suspensions of dbrB adenocarcinoma from dba mice were stored at $6\pm2°$ C. in a water-soluble extract of *Staphylococcus aureus* cells and in Tyrode's solution for periods of 2 to 24 days, the viability of the stored cells being tested at 48 and 72 hour intervals during this time by inoculation into dba mice. The percentage takes were reduced by storage in Tyrode's solution and were further reduced when the storage medium was *Staphylococcus aureus* extract. The latent period was longer in the animals receiving the suspensions stored in the bacterial extract than in those inoculated with the control suspensions stored in Tyrode's solution. The survival time was not greatly modified in any instance. The terminal size of the tumors was approximately the same as that of those developing in animals from suspensions which had not been exposed to low temperatures or to bacterial extract at low temperatures. Histologically, none of the experimental or control tumors differed regardless of the period of storage. No tumor regressions occurred. Animals that failed to develop tumors from suspensions of cells stored at low temperatures and in bacterial extracts were not immune when subsequently injected with fresh suspensions of the tumor.

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Effect of Beef Spleen Extract on Respiration of Normal Liver and dbrB Adenocarcinoma During Storage at 4° C.*

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The data in the present report on the respiration of tumor tissue following storage at 4° C. in the presence of beef spleen extract were assembled during a series of experiments to establish a method of assay of the activity of beef spleen extract which has been reported as beneficial in the treatment of human basal cell epithelioma (1, 2). Amersbach, Walter, and Sperti (1) reviewed the earlier work on animals in this field. Extracts of mouse, horse, and beef spleen were found to enhance the respiration and depress the glycolysis of several mouse tumors (3) whereas extracts of other animal tissues similarly affected the metabolism of adenocarcinoma No. 63 and a methylcholanthrene-induced sarcoma (7). Extracts of beef spleen increased the respiration of normal rat liver and skin (4).

PROCEDURE

Female dba mice, 3 months old, were inoculated with dbrB tumor suspension into the groin. Two weeks later when the tumor was one-fourth the size of the animal, it was removed under light ether anesthesia and aseptic conditions, and macerated by passing through a fine mesh screen with a porcelain pestle. The pulp was suspended in twice its volume of Tyrode's solution and the suspension divided into 2 portions. To 1 was added one-third its volume of Tyrode's solution and to the other a similar volume of beef spleen extract (8), containing 142 mgm. of solids per ml. Due to dilution factors during preparation of the stock suspension the final concentration of beef spleen extract was approximately 35 mgm. per ml. and during the respiratory period it was 12 mgm. per ml. Both the tumor suspension in Tyrode's solution and the tumor suspension in beef spleen extract were stored at 4° C. for 12 days. At 48 hour intervals portions were removed and allowed to stand for 1 hour at room temperature prior to determination of the respiratory rate by the Warburg technic (5).

As a comparison for the study of the respiration of the dbrB mammary adenocarcinoma, liver was obtained from normal dba mice of the same age, prepared and stored in a manner similar to that employed for the tumor tissue. Prior to storage, in all except the first series of experiments, the respiratory rate of the tumor and liver tissues was determined within 30 minutes of removal from the animal and is designated in the tables as "0" day. Four series of experiments were performed, using a total of 77 tumor-bearing animals and 31 normal mice for the liver tissue. The determination of the respiratory rate of the tumor suspension and normal tissue was made at 37.5° C. in the Warburg respirometers. In each determination 4 of the flasks were used for tumor tissue, 4 for normal liver tissue, and 2 additional flasks served as barometric controls.

In the inner well of each flask was 0.2 ml. of 1 *N* potassium hydroxide and the outer chamber contained 2 ml. of Ringer-phosphate-glucose solution, pH 7.2. Of the 4 experimental flasks for tumor tissue, 2 contained 1 ml. each of tumor suspension that had been stored in Tyrode's solution and the other 2, suspension that had been stored in beef spleen extract. Similarly, of the 4 experimental flasks for normal tissue, 2 contained 1 ml. each of liver suspension stored in Tyrode's solution and 2 contained 1 ml. each of suspension stored in beef spleen extract. Readings were taken at 60, 120 and 180 minutes. The suspensions were then dried overnight in weighed crucibles in a drying oven at 70° C. The calculated weight of the salt content of the Ringer-phosphate-glucose solution was subtracted from the dry weight of the tissue. The cubic mm. of oxygen per hour per mgm. of dry weight (QO_2) for the first and second 60-minute intervals and the total cu. mm. of oxygen per 3 hours per mgm. of dry weight were calculated. The values in the tables represent the average of duplicate determinations. When the experimental error was greater than 10 per cent the data were discarded.

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RESULTS

The data on the oxygen consumption of the dbrB tumor from dba mice and of normal dba mouse liver after storage at 4° C. for 2 to 12 days are presented in Tables I, II and III. This information represents the average of 4 series of experiments performed, using tumor-bearing animals and normal mice for the liver tissue. Examination of these tables show that: (a) during storage at 4° C. the respiration of both tumor and normal liver progressively decreased; (b) the respiratory rate of the tumor tissue was higher than that of normal liver tissue; (c) the respiratory rate for the tumor tissue was maintained during the second 60 minutes of the experimental period of 3 hours, while that of the liver was not; (d) the QO_2 for the third experimental hour was inconsistent for both the tumor and normal tissue; (e) in the presence of the beef spleen extract the QO_2 as well as the total oxygen uptake of the tumor was lower than in the presence of Tyrode's solution; (f) conversely, that of the liver was higher after storage in the beef spleen extract than after storage in Tyrode's solution.

TABLE I: AVERAGE Q_{O_2} FOR FIRST 60 MINUTES OF DBRB TUMOR AND NORMAL DBA LIVER STORED AT 4° C. FOR 2 TO 12 DAYS IN TYRODE'S SOLUTION AND BEEF SPLEEN EXTRACT

Series I to IV	DAYS IN STORAGE 0	2	4	6	8	10	12
	dbrB TUMOR						
Tyrode's solution	2.55	2.26	1.74	1.15	0.71	0.59	0.49
Beef spleen extract	1.77	1.71	1.10	0.79	0.54	0.47	0.43
	NORMAL LIVER TISSUE						
Tyrode's solution	1.45	0.95	0.50	0.46	0.30	0.30	0.25
Beef spleen extract	1.43	1.27	0.90	0.70	0.56	0.47	0.54

TABLE II: AVERAGE Q_{O_2} FOR SECOND HOUR OF DBRB TUMOR AND NORMAL DBA LIVER STORED AT 4° C. FOR 2 TO 12 DAYS IN TYRODE'S SOLUTION AND BEEF SPLEEN EXTRACT

Series I to IV	DAYS IN STORAGE 0	2	4	6	8	10	12
	dbrB TUMOR						
Tyrode's solution	2.27	2.10	1.25	0.85	0.69	0.55	0.49
Beef spleen extract	1.62	1.59	1.07	0.64	0.45	0.53	0.68
	NORMAL LIVER TISSUE						
Tyrode's solution	0.42	0.17	0.15	0.14	0.13	0.28	0.14
Beef spleen extract	0.49	0.32	0.25	0.22	0.12	0.20	0.24

TABLE III: AVERAGE OF TOTAL OXYGEN UPTAKE (CU. MM. PER MGM. OF TISSUE) OF STORED DBRB TUMOR AND NORMAL MOUSE LIVER TISSUE IN TYRODE'S SOLUTION AND BEEF SPLEEN EXTRACT OVER A PERIOD OF 3 HOURS

Series I to IV	DAYS IN STORAGE 0	2	4	6	8	10	12
	dbrB TUMOR						
Tyrode's solution	8.14	6.66	5.07	3.41	3.00	2.40	2.16
Beef spleen extract	5.97	5.69	4.18	2.58	2.21	2.14	2.44
	NORMAL LIVER TISSUE						
Tyrode's solution	2.14	1.33	0.82	0.80	0.53	0.55	0.47
Beef spleen extract	2.25	1.75	1.33	1.08	0.95	0.79	1.06

DISCUSSION

The data presented on the respiration of dbrB adenocarcinoma from dba mice, and of liver from normal dba mice, confirm the observations of other investigators that some tumor tissues may have a higher respiratory rate than certain normal tissues, as well as the data of Cook and Walter (4) who reported that the respiration of rat liver was increased in the presence of beef spleen extract. In contrast to the lowered respiratory rate of the dbrB adenocarcinoma in these experiments in the presence of spleen extract, Büngeler (3) reported an increased respiratory rate for Ehrlich's adenocarcinoma, a transplantable sarcoma and a tar carcinoma. Moreover, Schroeder and Cook (7) using heterozygous strains of mice and rats, found that the respiratory rate of adenocarcinoma No. 63 and methylcholanthrene-induced sarcomas was raised in the presence of extract of mouse embryos and of organs of the mouse exclusive of the spleen.

The effect of the spleen extract on respiration of dbrB adenocarcinoma in these experiments is of interest in view of the results obtained by Macfarlane, Schmock and Nadeau (6) with implantation of suspensions of the same tumor stored in spleen extract at 4 to 7° C. for as long as 8 days.

SUMMARY

The oxygen consumption of drbB adenocarcinoma from dba mice and of liver from normal dba mice was measured in the Warburg respirometer at 48 hour intervals during a period of 12 days of storage at 4° C. in Tyrode's solution and in beef spleen extract. The respiratory rate of both tumor and liver tissue progressively decreased during storage, that of the adenocarcinoma being consistently higher than that of the normal liver. Tumors stored in beef spleen extract had a lower respiratory rate than tumors stored in Tyrode's solution, while normal liver stored in beef spleen extract had a higher rate of respiration than liver stored in Tyrode's solution. The depression of respiration with the beef spleen extract of dbrB tumor from dba mice is in contrast with the stimulation reported by others using spleen extract and other types of tumors in heterozygous strains of rodents.

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The Response of Normal and Malignant Lymphoid Tissue to Methyl-Bis-(β-Chloroethyl)amine and Ethyl Carbamate (Urethane) in Adrenalectomized and Non-Adrenalectomized Mice *

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In a recent review Gilman and Philips (3) reported that nitrogen mustards in general have a depressant cellular effect which is most striking in tissues containing rapidly proliferating cells. Ethyl carbamate (urethane) is another drug that acts on dividing cells. Guyer and Claus (4) have shown that there is a marked reduction in the number of mitotic figures in the corneas of urethane-treated rats and mice. Colchicine effects an inhibition of cellular growth primarily as a result of mitotic arrest in the metaphase. These 3 agents have found clinical and laboratory usefulness in leukemia, presumably due to the cellular effects mentioned above.

The studies of Selye (8) clearly indicate that stimulation of the adrenal gland, as seen in the "alarm" reaction, plays a role in lymphoid regression. It has, therefore, been natural to assume that possibly the lymphoid regression produced by the above chemicals is at least in part a result of direct or indirect adrenal stimulation resulting from drug administration. It has been shown by Bass and Freeman (1) that a nonspecific stimulus induced by intraperitoneal administration of ethyl alcohol results in regression of malignant lymphoid tissue. On the other hand, Karnofsky, Graef, and Smith (5) have shown that in the adrenalectomized rat, nitrogen mustards produce reduction in the size of the spleen, thymus, and lymph nodes. The adrenal hypertrophy observed by Ludewig and Chanutin (6) studying nitrogen mustards and by Murphy and Sturm (7) studying urethane in rats, therefore, may be interpreted as either unrelated to the lymphoid regression produced by these agents or as of only minor significance.

The present investigation was undertaken to study further: (1) the effect of these agents on normal and malignant lymphoid tissue and (2) the role of the adrenal glands in producing lymphoid regression in urethane and nitrogen mustard-treated mice.

EXPERIMENTAL

Comparative effects of colchicine, urethane and methyl-bis (β-chloroethyl) amine hydrocloride.[1] C3H mice bearing transplanted 6C3HED lymphomas were used for our study. This tumor has proved very satisfactory as we have not observed spontaneous regression in our control animals. Therefore, an evaluation of therapy on small groups of mice has been possible, as any regression or maintained inhibition could be considered a result of medication. The dose selected for each drug was one which was lethal to approximately 10 per cent of the animals treated. This dosage assured a maximum therapeutic effect.

Fig. 1 gives the results of therapy with urethane, colchicine, and methyl-bis-(β-chloroethyl)-amine (HN_2), both alone and in combination, on tumor growth. Change in tumor size is indicated as the difference between the size of the tumor on any given day and that at the time therapy was begun. Tumor size was arbitrarily considered to be one-half the length plus the width expressed in millimeters. The order of increasing activity for the individual drugs tested is: HN_2, urethane, and colchicine. The regression obtained with HN_2 plus colchicine is no greater than with colchicine alone. This is to be expected since the regression resulting from colchicine therapy alone is so striking. Although the difference between the results with urethane alone and with urethane plus HN_2 is not great, there seemed to be a slight potentiation of action.

When C3H mice became unavailable, we continued our studies on Rockland white mice. The response of the normal lymphocyte of the spleen and thymus under various conditions of therapy

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[1] Source of HN_2—Methyl bis (β chloroethyl) amine hydrochloride was obtained from Merck and Co., Rahway, N. J., through the courtesy of Dr. C. P. Rhoads, Chairman, Committee on Growth, National Research Council.

[2] These mice were raised in our laboratory from breeders obtained from Carworth Farms.

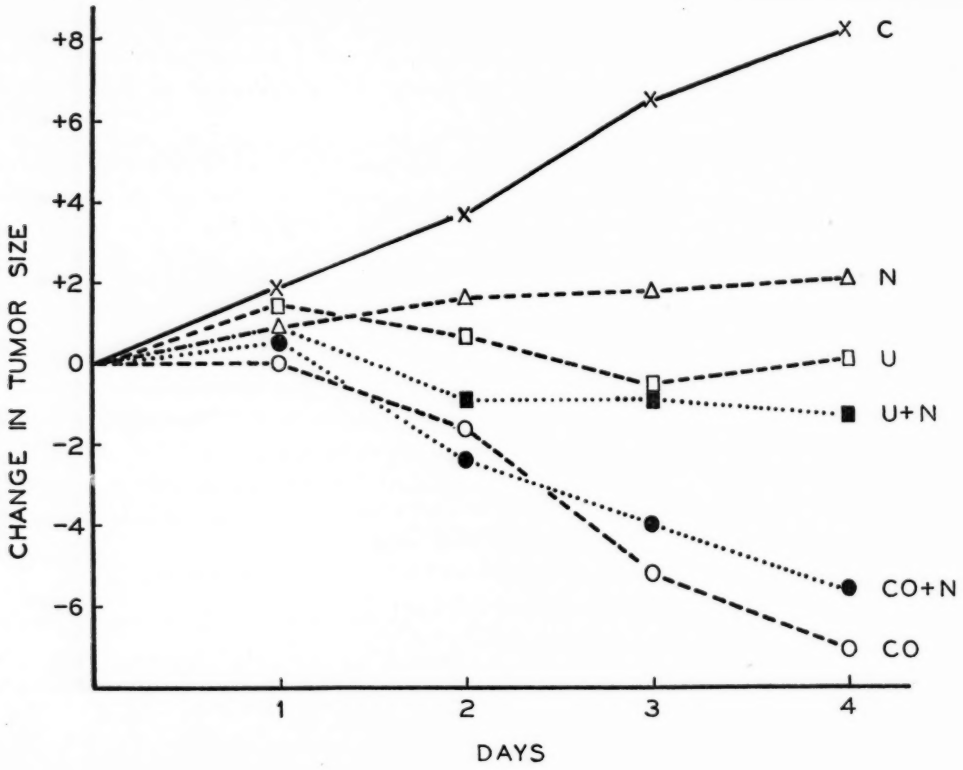

FIG. 1.—Growth curves of 6C3HED tumors in C3H mice of both sexes. C = Solvent controls for groups on combined therapy. N = Nitrogen mustard dissolved in isotonic sodium chloride 2 mgm. per kgm. administered on alternate days. U = urethane, U.S.P., dissolved in distilled water, 100 mgm. per 100 gm. of body weight daily. U+N = combined dosage as given above. HN_2 given 8 hours after urethane. Co = colchicine, U.S.P., dissolved in distilled water, 0.75 mgm. per kgm. daily. Co+N = colchicine plus nitrogen mustard in dosage as given above. HN_2 was given 8 hours after colchicine. All injections were made intraperitoneally. Number of animals: C = 18, N = 31, U = 10, U+N = 18, Co = 10, Co+N = 15.

was investigated. Adult male mice of approximately the same age were selected. All animals were sacrificed by severing the cervical cord after 4 days of therapy. Wet weights of thymus and spleen were recorded. Adrenal weights, also obtained, will be discussed later. Treatment schedules were the same as those employed for the C3H mice, but the dosage was increased to correspond with the higher LD50 of the respective agents for white mice.

Fig. 2 shows the results obtained. The spleen and thymus of animals treated with urethane and HN_2 were significantly smaller than those of the controls. Organ weights after combined therapy averaged slightly less than after the administration of a single agent.

To determine whether combined therapy would be as effective when nonlethal doses were used, a group of littermates of the Akm strain[2] were similarly studied.

Fig. 3 shows that each mouse receiving combined therapy has a smaller spleen and thymus than its littermates receiving either drug alone. Although there were no deaths at the dosages employed, organ size with the combined therapy was generally equal to, if not greater than, that obtained with lethal doses of either urethane or HN_2 alone. This observation suggests the advantages of using these drugs in combination. By such a technic regression of normal lymphoid tissue can be obtained without danger of lethality.

Effects of urethane and HN_2 in adrenalectomized animals—It has been reported by Karnofsky and his co-workers (5) that HN_2 is effective in reducing the size of the lymph nodes, thymus and spleen in adrenalectomized rats. Since no data was presented on the malignant lymphocyte changes of HN_2-treated animals following adrenalectomy, we have undertaken to compare the effect of both urethane and HN_2 on malignant and normal lymphoid tissue in adrenalectomized mice.

Both Rockland white and F_1 C3H hybrid mice were used in this study. The Rockland mice were given 0.025 mgm. per mouse of desoxycorticosterone acetate in sesame oil subcutaneously on the day of operation; beginning on the second day they

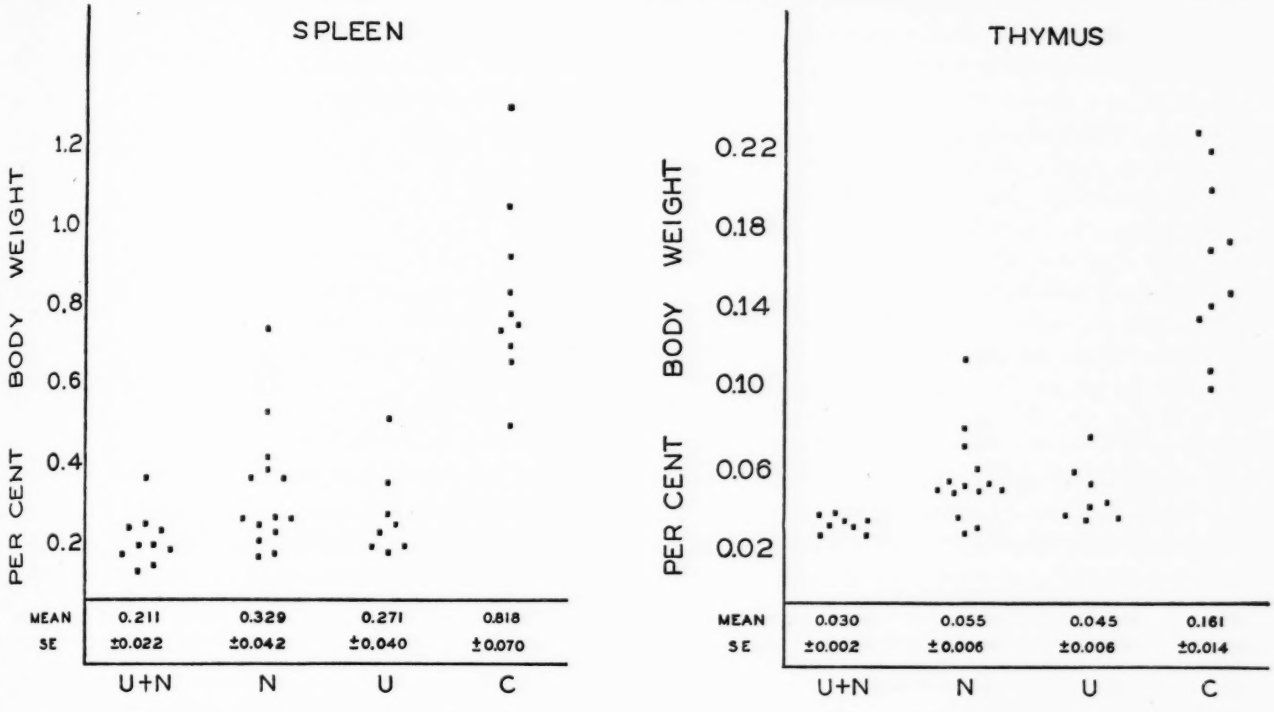

FIG. 2.—Thymus and spleen weights following nitrogen mustard and urethane therapy. Rockland Farms white adult male mice were treated as outlined below and were sacrificed at end of fourth day of therapy. C = solvent controls for group U+N. U = urethane, U.S.P., dissolved in distilled water, 152 mgm. per 100 gm. of body weight daily for 2 days, followed by 100 mgm. per 100 gm. daily for 2 days. N = nitrogen mustard dissolved in isotonic sodium chloride 6 mgm. per kgm. on first day of therapy and 3 mgm. per kgm. on third day. U+N = urethane and nitrogen mustard in quantities stated for individual U and N groups. HN_2 was administered 8 hours after injection of urethane. All injections were made intraperitoneally. (SE = standard error).

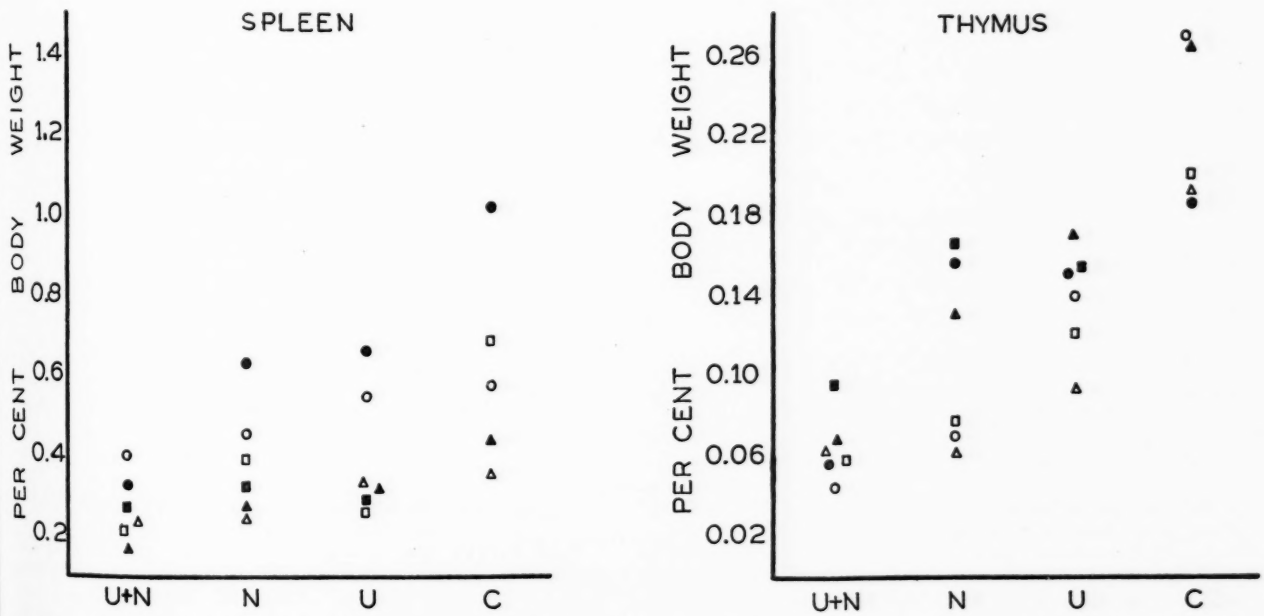

FIG. 3.—Effect of urethane and nitrogen mustard therapy on spleen and thymus weight in 6 week old Akm strain mice. Solid symbols represent female mice; open symbols male mice. Each litter is represented by a different symbol. C = solvent control for U+N group. U = urethane dissolved in distilled water, dose 50 mgm. per 100 gm. of body weight. N = nitrogen mustard dissolved in isotonic sodium chloride, 1.5 mgm. per kgm. on alternate days. U+N = urethane and mustard in quantities stated for individual U and N groups. HN_2 was given 8 hours after urethane. All injections were made intraperitoneally. Animals were sacrificed after 4 days of therapy.

were maintained on 0.9 per cent NaCl as the drinking water. The C3H hybrids were not given desoxycorticosterone acetate and were maintained on saline as a source of fluid from the time of operation. Adrenalectomy was performed under ether anesthesia. Although immediate operative mortality was negligible, deaths were frequently encountered during the experimental period.

Fig. 4 A shows that tumor inhibition occurs in adrenalectomized mice treated with urethane and HN_2; this is evident even with the reduced drug dosages which are necessary with such animals. Fig. 4B shows the decreases in spleen size observed in urethane and HN_2-treated Rockland white and C3H hybrid mice. Although the differentials are not marked with this lower dosage, it is clear that the same pattern of organ response as observed in nonadrenalectomized mice is obtained. From the data presented it is evident that both urethane and HN_2 are effective "lympholytic" agents in adrenalectomized animals.

Ludewig and Chanutin (6) have shown that there is an enlargement of the adrenal gland in HN_2-treated rats. This is associated with a decrease in cholesterol ester and an increase in the protein and water content of the glands. Results obtained with mice in this laboratory are shown in Table I. Mice treated with both HN_2 and urethane show an increase in wet and dry adrenal weight as compared with the controls. Although chemical analyses were not performed, the data presented suggest that the adrenal weight-response to urethane administration may be on the same basis as that following HN_2 administration.

Since atrophy of malignant and normal lymphoid tissue occurs when adrenalectomized mice are treated with urethane and HN_2, the adrenal hypertrophy resulting from administration of these

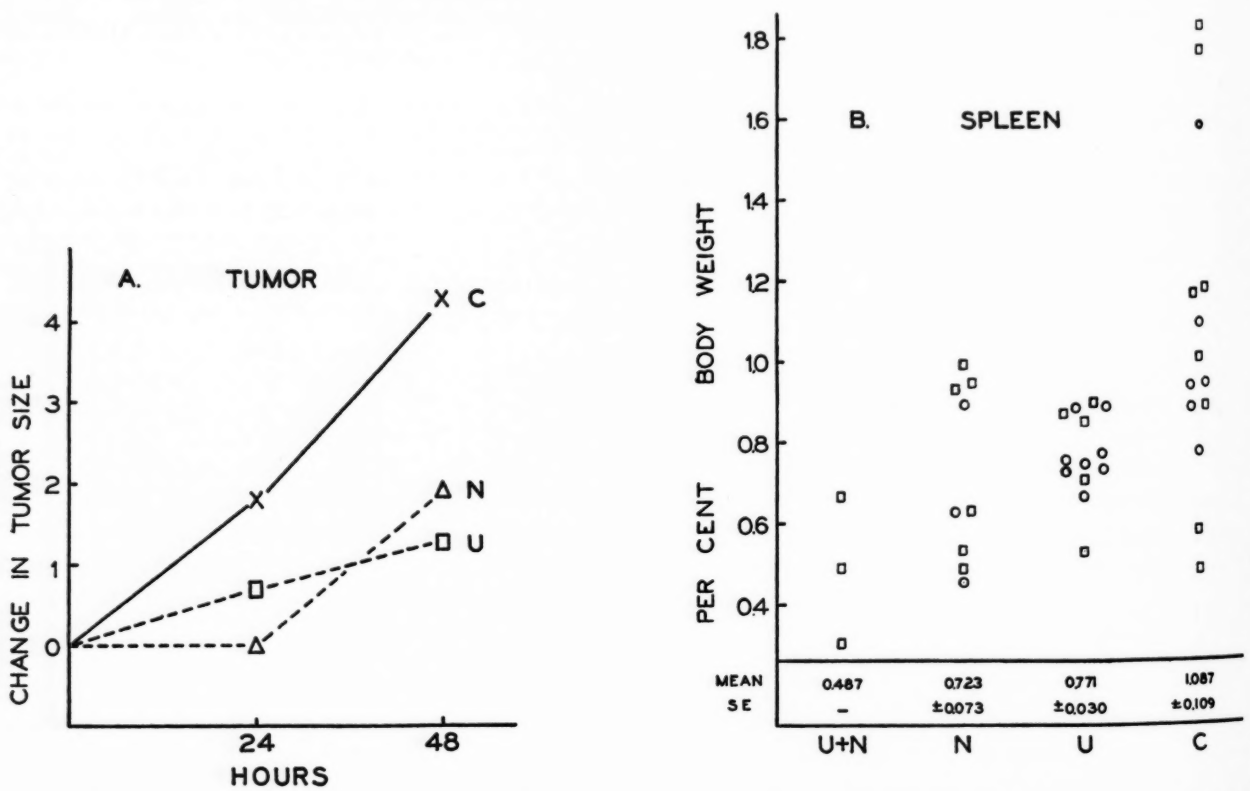

FIG. 4—Tumor and spleen response to urethane and nitrogen mustard in adrenalectomized male mice.

A. C = solvent adrenalectomized controls received daily injections of the water, and single injection of isotonic sodium chloride on first day of therapy. N = nitrogen mustard dissolved in isotonic sodium chloride. One dose of 1.5 mgm. per kgm. was administered on first day of experiment. U = urethane, U.S.P., dissolved in distilled water 75 mgm. per 100 gm. daily. All injections were made intraperitoneally. Number of animals: C = 5, N = 5, U = 7. C3H F_1 hybrid mice bearing 6C3HED tumors were used.

B. O—Adrenalectomized mice, male, C3H F_1 hybrid
□—Adrenalectomized mice, male, Rockland.

C3H hybrids were given same doses as in A except for an additional dose of HN_2 on the third day to the N group and to the U+N groups. Rockland white mice received 50 mgm. per 100 gm. of urethane daily and HN_2 1.5 mgm. per kgm. on the first and third day of experiment. The U+N group received both agents in quantities stated for the individual U and N groups. HN_2 was given 8 hours after the urethane. All injections were made intraperitoneally. Animals were sacrificed at end of fourth day of therapy. (SE = standard error).

agents cannot be taken as evidence that these chemicals have their major action through production of an "alarming stimulus."

Further evidence that the action of HN_2 and urethane is a direct one on the affected cell, is offered by Guyer and Claus (4), who have shown that nearly all mitotic forms can be eliminated in the corneas of rats and mice treated with urethane, and by Friedenwald and associates (2) who have shown that HN_2 acts on the corneal cells in the premitotic phase.

To determine whether cellular activity similar to that seen on the cells of the cornea was also evident in lymphoid tissue, the effect of injected urethane was studied.

The reduction of the mitotic activity in the spleen of a C3H mouse treated with urethane is shown in Fig. 5. Iron hematoxylin, which has an affinity for nuclear material of cells in stages of mitosis, was employed to demonstrate altered nuclear activity. It seems evident that the urethane-treated animal has few cells that are stained with this dye, whereas large amounts of the dye are taken up by the lymphocytes of the untreated animal. The stained cells are largely in the prophase stage of mitotic activity. This evidence indicates that the findings of Guyer and Claus for the mouse cornea also apply at least qualitatively to the mouse spleen. That nitrogen mustard has a direct action on blood-forming organs was reported in the review of Gilman and Philips. (3).

SUMMARY AND CONCLUSIONS

The response of normal and malignant lymphoid tissue to urethane and HN_2 administration has been studied in mice. Both urethane and HN_2 caused a retardation or regression of tumor growth. A comparable effect was demonstrated on normal lymphoid tissue, as determined by the organ weight of the spleen and thymus. The data indicate that a combination of both agents in non-toxic doses are as effective as either drug used alone in toxic dosages.

It has been clearly demonstrated that these agents are effective in adrenalectomized animals. The observations of Karnofsky and his group (5) on rats have been confirmed in mice and it has been further shown that urethane and HN_2 are capable of producing regression of a lymphoid tumor 6C3HED in adrenalectomized animals. Since hypertrophy of the adrenal gland occurred in mice treated with urethane and HN_2, one might suspect that the "alarm" reaction was at least partially responsible for the effects observed on lymphoid tissue. The results obtained in adrenalectomized animals, however, indicate that if the adrenal plays any role at all, it must be a very minor one.

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Table I

Experimental animal	Sex	Therapy*	Total dose mgm./kgm.	Number of animals	Weight of adrenal pairs mgm. per 100 gm. body weight: Wet weight mean ± standard error	Wet weight† mean
Rockland white mice	Male	Solvent control	....	10	20 ± 1.1	5.86
		Urethane	5040	8	30 ± 3.0‡	6.68
		Nitrogen mustard	9	14	29 ± 2.1‡	7.50
		Urethane + Nitrogen mustard	5040 + 9	10	34 ± 4.3‡	7.87
Carworth Akm strain mice	Male and female	Solvent control	....	12	19 ± 1.9	6.04
		Urethane	2000	17	25 ± 2.4	8.42
		Nitrogen mustard	3	17	26 ± 2.5	8.10
		Urethane + Nitrogen mustard	2000 + 3	17	28 ± 2.4‡	8.34

* Urethane was dissolved in distilled water; nitrogen mustard in isotonic saline.
‡ Weights are significantly greater than respective controls upon statistical analysis.
† Mean = $\frac{\text{total weight of pooled adrenals}}{\text{number of adrenal pairs in pool.}}$

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DESCRIPTION OF FIGURE 5

FIG. 5—Photomicrographs of spleens of Rockland white male mice. A. Normal mouse injected intraperitoneally with 0.23 ml. water. B. Normal mouse treated with one dose of urethane dissolved in water, 152 mgm. per 100 gm. of body weight intraperitoneally. Both animals were sacrificed 8 hours after injections were made. Iron hematoxylin stain. Mag. × 165.

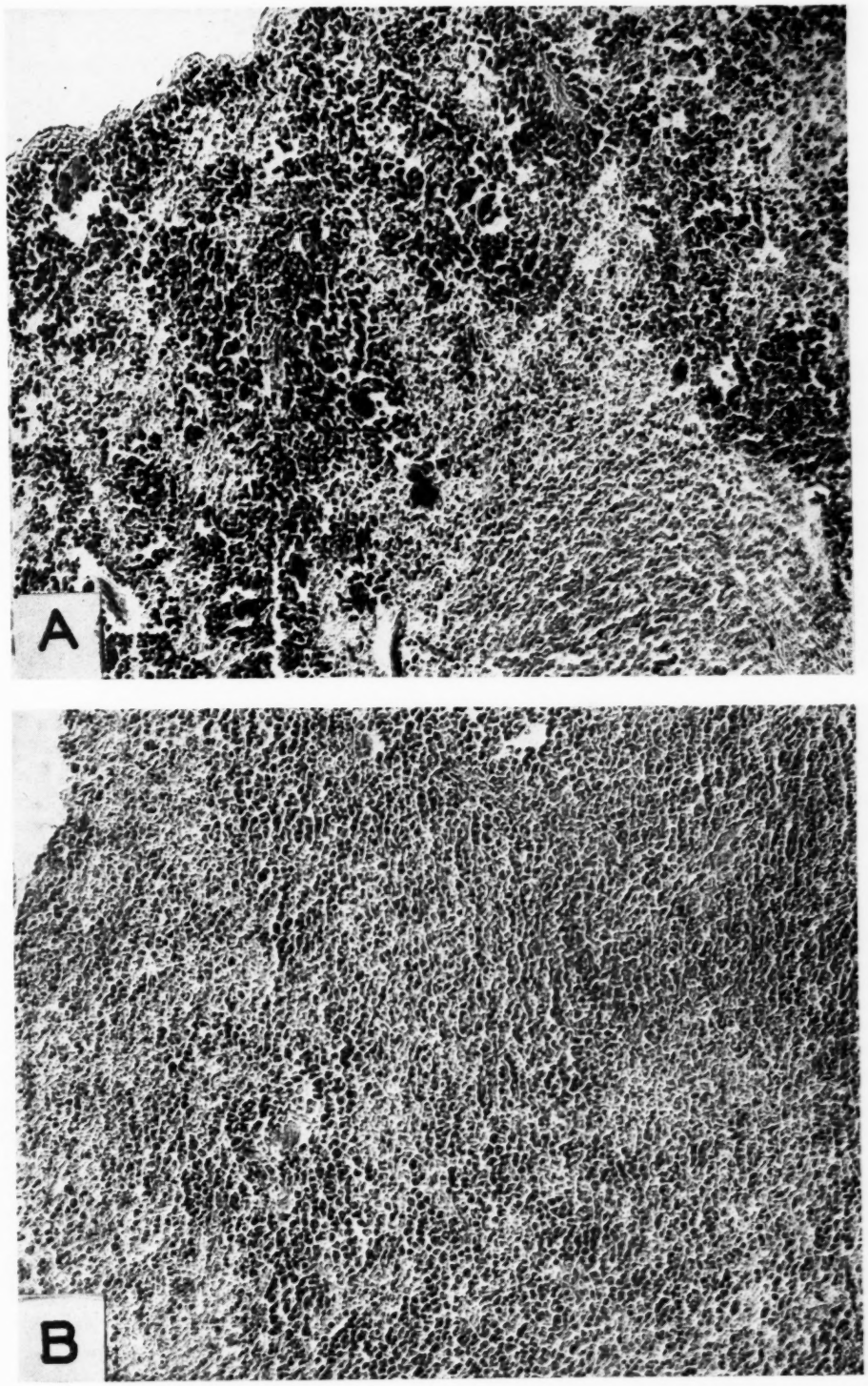

FIG. 5

Book Reviews

EXPERIMENTAL EMBRYOLOGY. M. W. Woerdeman and Chr. P. Raven. Monographs on the Progress of Research in Holland during the War. New York: Elsevier Publishing Company. 1946.

This report by two well known experimental embryologists recounts the result of work carried on in their laboratories during the German occupation. Woerdeman is professor of anatomy at the University of Amsterdam; Raven holds a similar post at the University of Utrecht.

Concise accounts are given of 19 experiments on amphibian embryos. Among these is a study on the influence of x-rays on blastulæ of the axolotl. The dosage used varied from 100 r to 1,000 r; quantities of radiation above 200 r bring about rapid death of the embryos. Even 100 r and 200 r produce injury to the organizer and the products of its inductive activity. This injury is effected in a manner corresponding to Child's "gradient of susceptibility." The influence of carcinogenic carbohydrates on the development of amphibian larvae was found to support the results of investigators working with adult tissues; *viz,* low concentrations accelerated the growth rate whereas higher concentrations depressed it. Provisional experiments have been carried out to determine the usefulness of the clawed toad *Xenopus laevis* for embryological studies. This animal, now widely used in clinical laboratories for pregnancy tests, is readily reared. Of great significance is the fact that during the blastula and gastrula stages the vitelline membrane can be readily torn off with the aid of watch-makers forceps. In this they offer a distinct advantage over other anurans in which the membrane cannot be separated until the neurula stage has been reached, greatly decreasing their usefulness as experimental material.

Work with chick embryos was limited to two experiments. One attempts an elucidation of the problem concerning the etiology of extra-gonadal teratomas. It is assumed that the primary gonocytes (primordial germ cells) migrate to the primordium of the gonads from the germinal crescent anterior to the head fold. It is suggested that since many teratomas develop dorsally in the median plane, the inductive effect of the chorda-mesoderm may incite the primary gonocytes to develop. The gonads of young chick embryos were therefore cultured in the presence of chorda-mesoderm. War conditions interfered with the completion of this provocative study. Another series of experiments, likewise incomplete, sets out to answer the question whether the embryonic gonads secrete a hormone and whether this exercises an influence on the development of the genital system. The problem is being approached by grafting gonads of the same and of the other sex on the host, host and donor being of the same or different ages.

A number of papers are devoted to a comprehensive study of the development of the pond snail *Limnaea stagnalis.* The aim of these investigations is to gain better insight into the determination processes in animals with so-called mosaic development, *i.e.,* in those forms where determination of the parts of the germ has already been achieved at an early stage of development.

Although much of the work reported in this monograph is incomplete it represents an achievement in which the directors of the two laboratories may well take much pride.

A CONTRIBUTION TO THE KNOWLEDGE OF THE INFLUENCES OF GONADOTROPIC AND SEX HORMONES ON THE GONADS OF RATS. J. H. Gaarenstroom and S. E. De Jongh. Monographs on the Progress of Research in Holland during the War. New York and Amsterdam: Elsevier Publishing Company, Inc. 1946. 164 pages. Price, $3.00.

In this monograph the authors report the results of endoctrine research carried out energetically in Holland during five years of German occupation. The studies deal largely with the physiological effects of various hormone injections on the testes and ovaries of rats. The authors give evidence to indicate that many gonadotropic phenomena should be viewed in a new light. The book is illustrated with many tables and a few photomicrographs. The literature not available to them during the war is reviewed and pertinent references to the work of other investigators is incorporated in this monograph.

APPROACHES TO TUMOR THERAPY. A Symposium of Papers and Discussions on Various Aspects of Tumor Chemotherapy, Developed from the Summer Meetings of the Section on Chemistry of the American Association for the Advancement of Science at Gibson Island, Maryland, 1945-1946. Publication of the A.A.A.S. Lancaster: The Science Press. 1947, 442 pages.

The problem of tumor therapy is discussed under 6 main headings: I. Historical Introduction; II. Special Methodology; III. Nutritional Factors; IV. Bacterial Products; V. Nitrogen Mustards; and VI. Various Clinical Abstracts.

The first section, Historical Introduction, consists of a general review of cancer therapy by William H. Woglom. Each of the succeeding topics is covered by papers from several authors, and the material presented under Bacterial Products and Nitrogen Mustards is of particular interest.

The book suffers from the usual faults inherent in such a compilation, namely, lack of precise organization, irregular coverage, and irrelevant or discursive material in the discussions Nevertheless, it contains a wealth

of information in an important field of cancer research. While it will be useful to the investigator of neoplasia rather than to the clinician or medical student, it is valuable chiefly for the inclusion under one cover of vast related material for the convenience of those who work along similar lines of research.

PRACTICAL EMULSIONS. H. Bennett. Second Edition. Brooklyn: Chemical Publishing Co., Inc. 1947, xvi + 568 pages. Price $8.50.

This is a loosely knit collection of general information, special topics and specific formulas relating to the field of emulsions. The Preface to the first edition states: "this book . . . touches only lightly the theoretical aspects and concentrates on the art of making and applying emulsions. No attempt has been made to compile a complete record of all published work on emulsions." In the second edition the author has adhered to this thesis while adding a section (Part II, 86 pages) composed of contributions of various authors on the subject of industrial emulsions and emulsifying agents. Most of the book is devoted to lists, with references, of surface-active agents, emulsifying agents, emulsions, emulsion formulas, etc. Many of the references are to patents, catalogues of various companies, Chemical Formulary and to "Personal communication." The book is printed in pleasing type on paper of excellent quality. There are few typographical errors. The index of subject matter is well done.

Part I deals briefly with emulsion types, emulsifying agents, foams and frothing, dispersing and wetting agents, methods and equipment used in the preparation of emulsions, and stability factors in emulsions.

In Part II the subjects, discussed by experts in their respective fields, include Lecithin as an Emulsifying Agent; Pectin as an Emulsifier; Pectin Emulsions and Ointments; Polyhydric Alcohol Compounds; Soap the Basic Industrial Emulsifier; Surface-Active Agents as Germicides; Cosmetic Emulsions; Dye Emulsions; Emulsion Paints; Food Emulsions; Synthetic Rubber Latex and Emulsions in Leather Manufacturing.

Part III lists 926 emulsion formulas covering many fields of application. The author has been active in developing more than two-thirds of these formulas; most of which have already been published in *Chemical Formulary*. Chief among them are polish emulsions, with 39 formulas for automobile polish alone. Under cosmetic and drug emulsions, are agricultural sprays, cutting oils, cleaners and soaps, and many others.

Although extremely useful, the value of the information contained in the book is limited by the fact that no indication is given as to how changes in composition in the various formulas affect the properties of the products.

Announcement of Availability of Grants and Fellowships

The Committee on Growth of the National Research Council, Acting for the American Cancer Society, is entertaining applications for grants and fellowships. Applications for extension of existing Grants in Cancer Research will be received until 1 October, applications for new grants until 1 November. Final decision on applications submitted during this period will be made in most cases soon after 1 February. Grants approved at this time ordinarily will become effective 1 July, 1949.

Fellowship applications may be submitted at any time. Those received prior to 1 November will be acted upon by the Committee on Growth in December. Those received between 1 November and 1 March will be acted upon in April. Fellowships ordinarily will begin 1 July though this date may be varied at the request of the applicant. During the past year the American Cancer Society, Inc., on recommendation of the Committee on Growth has approved research grants and fellowships totaling over $2,000,000.

Communications regarding grants and fellowships should be addressed to Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, N. W., Washington 25, D. C.